BIOCHEMISTRY OF TRACE ELEMENTS

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General Introduction

Trace elements occupy a unique place as dietary constituents which profoundly influence animal, plant and microbial metabolism. The requirement for these elements has been found to be very low, though their essentiality has been proved beyond doubt. As early as 1860, Pasteur has shown that yeast will grow only when the medium is supplemented with inorganic compounds. The importance of inorganic salts in the growth of plants and animals has been realised by the pioneering contributions of Sachs and Knop and Osborne and Mendel respectively.

Metals like iron, copper, molybdenum, zinc, manganese, cobalt and a few others have a wide variety of functions to perform in the living cell. They form part of several proteins and thus govern the structure and function of metalloproteins. In addition, metal ions have activating or inhibiting effects on the activities of several enzymes. Vitamin B_{12} contains cobalt and the hormones insulin and thyroxine contain zinc and iodine respectively. However, zinc is introduced into insulin during crystallization of the hormone. Metals have been detected to be present in nucleic acids (1) though their role in nucleic acid structure and function is not clear

An impressive amount of data has accumulated on the possible sites of trace element function. But still, knowledge has to be gained especially on the mechanisms involved in the transport of these metals to specific sites, since the competing metabolites are many and are of different molecular

Two approaches are generally available in a study of the metabolism of trace elements. One is to create a straight deficiency of the concerned

trace element in the experimental organism and study the metabolic derangements that are produced. The very low requirement of the trace element makes it possible that a slight excess over the optimal requirement assumes a toxic level and thus the study of trace element toxicity forms the second approach to this problem. These approaches are obviously the results of the diagnosis of certain types of animal and plant diseases as due to a trace element deficiency or toxicity. To mention only a few examples, chlorosis in plants has been attributed to a gross iron deficiency. Molybdenum deficiency in cauliflower causes the characterestic 'whiptail' disease. Pining disease in sheep has been attributed to cobalt deficiency. Selenium toxicity causes alkali disease in cattle. An inadequate intake or utilization of iodide causes goiter in man.

The deficiency or toxicity of a trace element can cause characterestic lesions in the experimental organism as well as create complex effects. Aser Rothstein (2) has discussed the situation arising out of toxicological studies and concludes that the "toxic chemical substances act ultimately by chemical reactions with biochemical substances in cells. In consequence, a cellular function is disturbed which manifests itself in an observed toxicological response in the animal. The chain of events between the original chemical insult and the final observed response may be so very complicated that one might seriously doubt that toxicological actions can ever be tracked to initial chemical events. Fortunately, they sometimes can. All too often, however, the limiting factor is the fund of knowledge concerning those normal patterns of cellular function at the chemical level, which can be disturbed by the toxic agents. For this reason, toxicological studies at the cellular level are doubly useful, leading not only to information concerning the mechanism of toxic response, but also to fundamental biological

information concerning cellular function". It is the premise of the present thesis that heavy metals can serve this function. Specific cases of cobalt and nickel toxicities have been studied in the mold Neurospora crassa and the thesis is advanced that such a study has led to :

1. An understanding of some aspects of normal iron metabolism of this organism.
2. An appreciation of the subtler differences in the toxicological responses evoked by closely related elements like cobalt and nickel despite a gross similarity in overall action.

The literature pertaining to the available information on the role of trace elements in metabolism and instances of ion antagonisms have been very well discussed (3,4,5). In the following pages, the literature pertaining to the effects of cobalt and nickel toxicities as well as iron deficiency, and the metabolic implications arisisng out of these have been briefly reviewed. The review has been restricted for the part to studies on microorganisms.

Cobalt and nickel

The relative toxicities of the trace elements like cobalt, nickel and zinc have been tested in filamentous fungi and the first two have been recognized to be more toxic than zinc (6). The concentrations of cobalt necessary to kill the cells of bacteria, yeast or paramecia after a given period of exposure have been reported by different workers (7,8, 9). Bedford (10) has reported that the toxic limit of cobalt for niger is around 1500 ; The toxicities of cobalt, copper, zinc and have been investigated in various Lactobacilli by Svec (11). In various other bacteria cobalt toxicity has been studied by Schade (12) and the

inhibiting concentration has been found to depend on (i) the sensitivity of the individual strain of bacterium (ii) the number of cells per ml used as inoculum and (iii) the constituents of the growth medium. Of 17 amino acids tested for counteraction, cysteine and histidine have been found to be be capable of overcoming growth inhibition due to cobalt. In a study of the uptake of cobalt by Proteus vulgaris (13) it has been found that magnesium is particularly effective in diminishing the cobalt-combining capacity of cells.

The first detailed report on heavy metal ion antagonism is by Abelson and Aldous (14). These authors have shown in the case of Aspergillus niger, Escherichia coli, Aerobacter aerogenes and Torulopsis utilis that toxic levels of cobalt, nickel, cadmium, zinc and manganese interfere with the utilization of magnesium. The toxic level of the heavy metal has been found to be determined by the magnesium status of the medium and magnesium in turn controls the uptake of some toxic metals by a mechanism presumably involving competition at the site of binding. Subsequently, Lavollay and his associates (15,16) have observed a similar competitive zinc-magnesium antagonism in Aspergillus niger. Ion antagonism studies have also been carried out in lactic acid bacteria by Macleod and Snell (17). It has been presumed that in zinc toxicity a catalytically inactive zinc-protein is formed and the counteracting ions displace zinc from the protein to form catalytically active protein.

The phenomenon of chlorosis in plants is a well documented phenomenon. This is primarily due to iron deficiency and can be induced non-specifically by the heavy metals cobalt, nickel, zinc, copper, manganese and cadmium at toxic levels (18). However, there are quantitative differences in the effects produced; further, differences exist in the

of various plants to the same heavy metal (19). However, this conditioned chlorosis can be corrected in almost all the cases by painting the leaves with ferrous sulphate solution (20). It has been possible to analyse the gross effects produced into subtler details as is illustrated by studies on nickel toxicity in oat plants (21,22, 23). Two types of effects have been noticed: (i) specific effects of the metal (ii) chlorosis due to induced iron deficiency. Interestingly, it has been observed that the leaf necrosis produced by nickel cannot be corrected by iron whereas chlorosis will respond to iron treatment. The necrotic areas are depleted in iron and magnesium though the tissue contents of these elements remain the same(24). Another instance of metal ion interrelationship has been observed in germinating seedlings of Phaseolus radiatus where magnesium has been found to counteract growth inhibition completely in zinc and partially in cobalt

A detailed analysis of trace element interrelationships in cobalt, nickel and zinc toxicities in the molds Aspergillus niger and Neurospora crassa has been carried out by Adiga, Sivaramasastry, Venkatasubramanyam and Sarma (26, 27) and the results may be reviewed here. In Aspergillus niger the toxic effects of these heavy metals include growth inhibition and inhibitions of acid production and glucose utilization. Both iron and magnesium can counteract growth inhibition due to all the three heavy metals. However, in general, magnesium but not iron can counteract depressed levels of acid production and glucose utilization. On the basis of the results obtained it has been concluded that in this organism zinc toxicity corresponds to a conditoned magnesium deficiency and cobalt toxicity to a conditioned iron deficiency. Similar interrelationships have also been observed in Neurospora crassa where iron and magnesium have been found to counteract growth inhibition due to cobalt, nickel and zinc toxicities. The magnesium

concentration of the medium has been recognised to be an important factor here. Under conditions of marginal magnesium nutrition of the fungus, the toxic level of the heavy metal is considerably lowered and further, the iron level required to counteract the growth inhibition is also increased.

The toxic manifestations are not only characteristic of the particular heavy metal but are also species specific. In the insect larva st. studies on cobalt, nickel and zinc toxicities have that while iron is beneficial in all the three cases, magnesium is effective only in the cases of cobalt and nickel but not in zinc toxicity. Further, zinc toxicity in this organism can be counteracted by purines as well as by the corresponding nucleosides and nucleotides and the nucleic acids (RNA and DNA), which do not inhibit the accumulation of zinc by the larval tissues. Thus, interference with the nucleic acid metabolism appears to be a primary lesion in zinc toxicity in this organism, but such effects are not observed in cobalt and nickel toxicities (28).

Studies on the modes of counteraction of the heavy metal toxicities by iron and magnesium in Aspergillus niger(29) and Neurospora crassa (27) have revealed that magnesium inhibits the uptake of the toxic metal but iron does not significantly influence the toxic metal accumulation by the organisms indicating that its effects are intracellular in nature.

A cobalt-iron interrelationship has been observed riboflavin production by Candida guilliermondii (30). The inhibitory effect of iron on riboflavin production has been found to be counteracted by cobalt. However, cobalt does not inhibit total iron uptake by the organism but instead enhances it. But, cobalt has a depressing effect on the uptake of iron into the inner region of the yeast cell. Iron in turn

does not inhibit the cobalt uptake by the yeast.

A cobalt-iron antagonism has been demonstrated in Neurospora crassa at the enzyme level by Healy, Cheng and McElroy (31). Depressed levels of certain enzymes of the Krebs' cycle and cytochromes, as well as catalase and peroxidase have been observed under conditions of cobalt toxicity. Similar changes have been brought about in straight iron deficiency and the enzymic derangements caused by excess cobalt can be counteracted by iron. However, the growth inhibition due to excess cobalt cannot be counteracted even at the highest level of iron used. Nickel behaves like cobalt only in some respects. For example nickel reduces succinic dehydrogenase but doubles catalase activity. A depressing effect of cobalt on cytochromes and catalase activity has been observed in liquid cultures of Proteus vulgaris (32)

Instances of cobalt interference with heme synthesis have been known thus accounting for depressed levels of cytochromes and heme enzymes under conditions of cobalt toxicity. Studies on heme synthesis in Rhodopseudomonas spheroides (33) have revealed that cobalt inhibits synthesis of iron protoporphyrin from δ -aminolevulinic acid. Other metals like nickel, zinc, lead and molybdenum have no effect. It has also been observed that the formation of protoporphyrin itself is inhibited by cobalt suggesting that its locus of action may be at a stage before the insertion of iron into protoporphyrin. In preparations of rabbit bone marrow (34) cobalt has been found to inhibit incorporation of radioactive glycine into heme. Cobalt has also been found to decrease iron protoporphyrin in cultures of ~~Corynebacterium~~ Corynebacterium diphtheriae (35). Studies on the metal specificity of the iron-protoporphyrin chelating enzyme from rat liver have revealed that both iron and cobalt can be utilized by the enzyme in the synthesis of heme. Nickel and several other metals are not incorporated by the enzyme (36). The iron-protoporphyrin chelating enzyme from chicken erythrocytes appears different and it can utilize

cobalt only 2%, as well as iron (37). However, Johnson and Jones(38) have found that the enzymes from avian erythrocytes and thiobacillus X do incorporate iron, cobalt and zinc. The previous failure to detect cobalt incorporation into protoporphyrin has been attributed to the use of ethyl acetate to extract the cobalto-porphyrin formed during incubation. It has been found that cobalto-porphyrin is insoluble in this solvent. Recently, an enzyme which specifically incorporates cobalt into the porphyrin nucleus has been demonstrated in Clostridium tetanomorphum (39). It is evident that cobalt can act at more than one site in influencing heme synthesis. It can inhibit the synthesis of porphyrin nucleus as well as offer competition at the site of enzymic incorporation of iron into protoporphyrin, thus accounting for depressed levels of heme systems in cobalt

of cobalt and nickel have been found to bring about various other metabolic abnormalities and interfere with the metabolism of carbohydrates, nucleic acids and proteins as well. Such effects may be the result of direct heavy metal action or as a result of an interference with the functions of an essential trace element. Iron deficiency as well as cobalt and nickel additions have been found to increase the neurotoxin production by Shigella shigae, a phenomenon apparently not related to heme formation (40). An examination of the effects of organic acids on cobalt, nickel and zinc toxicities in Aspergillus niger have revealed that the adverse effects of the heavy metals on acid production and growth can be counteracted by citrate, malate, succinate, fumarate and pyruvate except in cobalt toxicity where the response to citrate is very partial and where succinate produces an added inhibition (41). It has been found in rat liver mitochondria that

the oxidation of the ketoacids of the Krebs' cycle is strongly inhibited by cobalt. It has been suggested that the action of cobalt, like that of arsenite, is due to complex formation between the ion and the dithiol form of lipoic acid, a coenzyme for keto acid dehydrogenation(42). Further studies on the mechanism of this inhibition phenomenon have revealed that the interaction of the reduced dehydrogenase complex with cobalt in the presence of air, leads to oxidative inactivation of the lipoyl coenzyme (43).

Levy, Skutch and Schade (44) on the basis of studies on nucleic acid turnover using P^{32} in Proteus vulgaris have concluded that the inhibitory effects of cobalt are related to an effect whereby ribonucleic acid leads to protein synthesis. Though protein synthesis is inhibited, the concentration of nucleic acids in inhibited cells is higher than in control cells. Cobalt at a concentration of 2×10^{-3}M has been found to inhibit growth and protein synthesis in yeast. The soluble nitrogen and nucleotide nitrogen are considerably higher in cobalt grown yeast than that grown under normal conditions(45). In Tetrahymena pyriformis it has been found that cobalt is more inhibitory to growth than equivalent concentrations of nickel. Excess cobalt results in a significantly increased RNA and DNA content per cell under certain conditions. Cobalt and nickel inhibit ribonuclease activity of homogenates prepared from the normal cultures (46). Deturk and Bernheim (47 have found in Psuedomonas aeruginosa that cobalt inhibits induction of urease and of the enzymes for the oxidation of putrescine, γ-aminobutyric acid and benzoate. In most instances the inhibitions are overcome by inorganic iron salts indicating that the two metals may be combining with the same receptors at the cellular level. Ballentine and Stephens(48) have detected the formation of stable cobalto-proteins in Neurospora crassa and have envisaged the possibility that cobalt forms an essential part of metabolizing

systems not only as a constituent of vitamin B_{12}, but possibly also as a part of enzyme systems in the cell. A cobalt-iron interaction on growth has been noted in Neurospora crassa and covariance analysis of data has been interpreted to mean that the metabolism of cobalt pertains directly to this element and not to iron. The incorporation of cobalt into the cytoplasm in place of iron by a metabolic mistake has been found to be improbable (49).

Iron deficiency effects and its metabolic implications

The studies so far reviewed clearly indicate that a major effect of heavy metal toxicities is a conditioned deficiency of an essential element. Especially, a direct cobalt-iron antagonism is demonstrable in several systems. Thus, the effects of direct iron deficiency, when clearly understood, can enable to get an insight into the possible sites of heavy metal action apart from an understanding of the metabolic processes requiring iron. The literature pertaining to iron deficiency effects with special reference to the studies on microorganisms has been briefly reviewed here.

Iron as a constituent of heme and as such in heme proteins has enormous functions to perform in the living cell. Thus, one effect of iron deficiency has been the accumulation of porphyrins with a concomitant fall in the levels of heme systems. Pappenheimer (50) has found that there is a parallelism between toxin and porphyrin production in the toxic filtrates of ~~Corynebacterium~~ Corynebacterium diphtheriae and suboptimal levels of iron increase the production of both. Still higher levels of iron increase the production of both, though growth itself continues to increase slightly (10-20%). For every 4 atoms of iron added to the culture medium (above that optimal for toxin production) 4 molecules of porphyrin and 1 of toxin disappear from

the culture filtrate. The results have been taken to suggest that the diphtheria toxin is the protein moiety of an iron containing respiratory enzyme. Clarke (51) has however disputed this hypothesis and finds that on the basis of their iron contents proto-hemin and iron coproporphyrin III are less active than ferrous sulphate in their effect on toxin yield. Townsley and Neilands (52) have shown that growing cultures or lysed cell preparations of Micrococcus lysodeikticus accumulate coproporphyrin III in iron deficiency. In Mycobacterium smegmatis it has been found that under conditions of iron deficiency the coproporphyrin is lower than normal upto the 4th day after which there is a steep rise (53). The accumulation of porphyrin in iron deficiency may be explained by the fact that heme is synthesised by the insertion of iron into the porphyrin nucleus. This process is believed to be an enzymic process as is evident from studies in animal systems.(54, 55). In microorganisms, probably, the first experimental evidence that protoporphyrin may be a precursor of heme is found in the studies of Granick and Gilder (56) which indicate the acility of

to utilize protoporphyrin in place of hemin as a growth factor. Invariably, the porphyrin that accumulates in iron deficiency has found to be coproporphyrin and it has been suggested that iron may be

for the conversion of coproporphyrin to protoporphyrin (57).

It is interesting to note that the heme biosynthetic pathway is very similar in animals, plants and microorganisms(58). The steps consist in the formation of δ-aminolevulinic acid, synthesis of protoporphyrin and incorporation of iron into protoporphyrin. Evidences are available to indicate that iron may be involved in the synthesis of δ-aminolevulinic acid in addition to the sites mentioned earlier. It has been demonstrated in chicken erythrocytes that α′-dipyridyl inhibits δ-amino levulinic acid

synthesis which is reversed by the addition of ferrous sulphate (59). Duesberg (60) has shown that there is a decreased porphyrin excretion in patients with iron deficiency. Thus, as is evident from the work of Pappenheimer (50) iron can significantly enhance porphyrin production and can also inhibit the synthesis of the same, depending on the concentration at which it is present. This together with the involvement of iron at the final step of heme synthesis amply substantiate to the regulatory role of iron in the maintenance of the activities of heme systems. Burnham and Lascelles (61) have detetected a significant inhibition of δ-aminolevulate synthetase by hemin in Rhodopseudomonas spheroides and have proposed that one mechanism for control of porphyrin synthesis in this organism may be through a negative feedback by hemin.

Waring and Werkman (62) have shown that iron-deficient Aerobacter aerogenes lack catalase. Similar results have been obtained with Nocardia opaca (63). Nicholas and Commissiong (64) have found in Neurospora crassa that the heme enzymes and 'TPNH diaphorase' become more active when the iron level is increased from deficiency to sufficiency. Healy, Cheng and McElroy (31) have recorded depressed levels of cytochromes, catalase and peroxidase in the same organism. Similar results have been obtained in Proteus vulgaris (32).

Another interesting feature in porphyrin synthesis is that the steps involved in the formation of protoporphyrin are the same whether the end product is cytochrome-hemin, hemoglobin- hemin or chlorophyll Thus, 'chlorosis' in plants can be traced to iron deficiency though chlorophyll itself does not contain this metal. A monoester of magnesium protoporphyrin has been isolated from etiolated barley leaves treated with

δ-amino levulinic acid and α,α'-dipyridyl (65). Lascelles (66) has found that cultures of Rhodopseudomonas spheroides accumulate large amounts of free porphyrin when grown in light on media deficient in iron salts. Iron salts have been found to completely suppress porphyrin formation from glycine and α-ketoglutarate, but increase the formation of bacteriochlorophyll and heme components. Iron does not suppress porphyrin synthesis from δ-aminolevulinic acid. It is suggested that porphyrin formation by Rhodopseudomonas spheroides is associated with the synthesis of bacteriochlorophyll and that iron is concerned in the conversion of porphyrins or derivatives into bacteriochlorophyll (67). Jones (68) recorded that in iron deficient cultures of Rhodopseudomonas spheroides there is also an appreciable increase in magnesium protoporphyrin-like material. It has been suggested that iron may be required for the enzymic transformations of the side chains of magnesium protoporphyrin monomethyl ester leading to the formation of chlorophyll, in addition to its activity in the early stages of porphyrin synthesis.

Apart from its involvement in heme and porphyrin synthesis, iron also regulates the activities of non-heme iron enzymes and brings about changes in the levels of enzymes which do not require iron for activity. Significant changes are also observed in the levels of key metabolites like the nucleic acids, protein, carbohydrate intermediates and lipids in iron deficiency. These changes clearly establish a generalised role for iron in metabolism apart from its specialized function such as in heme synthesis.

Healy, Cheng and McElroy (31) have found in Neurospora crassa that iron deficiency in this organism effects changes not only in heme

sites of iron involvement in salicylic acid formation from aromatic or a non-aromatic precursor such as shikimic acid. Bacteria from iron and zinc deficient cultures have been found to have a low RNA and DNA concentrations from the time of growth inhibition and possibly preceding it (77). In a detailed study of iron deficiency on the composition of Mycobacterium smegmatis, Winder and O'Hara (53) have found that the level of ribonucleic acid falls at the time of growth inhibition and there is a slight fall in deoxyribonucleic acid. The ribonucleic acid from the deficient culture has a normal base composition. The length of the bacteria increases several times during iron deficiency. The pyridine nucleotide levels decrease much earlier as compared to the normal culture. The fall in total lipids is not appreciable and phospholipids rise in metal deficiency. In Aerobacter aerogenes (62) and Nocardia opaca (63) it has been found that under conditions of iron deficiency the oxygen uptake with certain substrates is markedly affected. The presence of pyruvic acid in the iron deficient cultures has been taken to indicate that the pyruvic oxidase of the organism may be iron dependent. Iron deficiency in plants has been found to produce profound changes in the levels of Krebs' cycle intermediates. Chlorotic leaves have been found to contain more citric acid and always distinctly less malic or oxalic acids than green leaves (78). Mustard plants made chlorotic by iron deficiency have a high ratio of K/Ca and this ratio decreases as the concentration of iron in the nutrient solution is increased (79). These changes have been attributed to a depressed aconitase activity under conditions of iron deficiency. Citric/malic and K/Ca ratios have been assumed to be linked metabolically(80). Azotobacter agilis (A. vinelandii) has been found to accumulate fluorescent pigments in the culture fluid under iron deficient conditions. The

principal yellow compound attached to a yellow-green fluorescent chromophore has been identified to be a peptide containing the uncommon aminoacids such as homoserine and β-hydroxyaspartic acid. As a working hypothesis it has been suggested that the compound normally associated with an iron-protein may be released into the medium when the co-ordinating iron atom is unavailable. Alternatively, it can arise from a biosynthetic pathway that is interrupted by iron deficiency (81). Several strains of iron deficient have been found to accumulate cis-3-methyl-5 OH-pentene 2 enoic acid (82).

A striking advance has been made in the elucidation of the mechanism of iron transport in microorganisms as a result of the isolation of certain compounds under iron deficient conditions which have a specific binding affinity for iron. Iron deficient cultures of Ustilago sphaerogena been found to secrete several iron-binding compounds into the culture fluid, the major component of which has been isolated, characterised and referred to as ferrichrome A
has been isolated from the normal cells of Ustilago
discovery of these compounds has opened up new avenues of research in the elucidation of microbial iron transport, since it has been found that the occurrence of ferrichrome type compounds is fairly widespread in microorganisms. Compounds either containing iron or having a profound binding affinity for iron have been isolated from different microflora. Coprogen has been isolated from coprophytic fungi (85). Lyr (86) has isolated from horse dung 3 microbial species which actively synthesise ferrichrome factors. A survey of 32 common microbial species has shown that 10 of these produce coprogen-like substances (87). Terregens factor (88), having a profound binding affinity for iron has been isolated from Arthrobacter terregens. Aspergillus niger

when grown under iron deficient conditions has been found to secrete an iron-binding compound which has been identified to be ferrichrome. Compounds closely related to the ferrichromes have been isolated from Aspergillus and

um species and have been referred to as ferricrocin, ferrichrysin, ferrirubin and ferrihodin (89). Another related substance is isolated from mycobacteria (90). Yet another group of compounds are the ferrioxamines isolated from streptomyces (91).

The common features of all these compounds are (I) a strong binding affinity for Fe^{+++} (II) the presence of a polyhydroxamate structure at iron-binding site and (III) despite differences in gross structure, a mutual replaceability of one with another as a growth factor for organisms

As against these, another group of closely related compounds which are generally growth inhibitory and antagonise the effect of the former list of compounds are the ferrimycins, isolated from Streptomyces griseofl
A classification has been proposed for all these compounds which have the characteristic hydroxamate structure at the iron-binding site. The ferric polyhydroxamate class of compounds have been referred to as siderochromes with the growth promoting ones being designated as sideramines and the growth inhibitory as sideromycins (92). Compounds like albomycin (93) and grisein (94) have been included in the latter group. The table alongside indicates the different members belonging to the two types of siderochromes (95).

The siderochromes can also be differentiated as peptides and non-peptides (96). Where as the ferrichromes can be considered as cyclic peptides (97), the ferrioxamines do not possess a typical peptide bond. An example of a non-siderochrome excreted under conditions of iron deficiency

Siderochrome

Sideramines Sideromycins

(a) From Actinomyces

Ferrioxamins

Nocardamin (identified to be the same as ferrioxamin E)

(b) From Fungi

Coprogen	Grisein
Ferrichrome	Albomycin group
Ferrichrysin	Ferrimycin A_1 , A_2 and B
Ferricrocin	Succinimycin
Ferrihodin	LA 5352
Ferrirubin	LA 5937

(c) From Bacteria

Terregens factor

Siderochrome without biological activity

Ferrichrome A.

has been the secretion of 2,3-dihydroxy benzoyl glycine (itoic acid) by Bacillus subtilis (99).

A great deal of work has been carried out on the structural elucidation of the siderochromes. Ferrichrome has been shown to contain

glycine and the unique amino acid δ -N-hydroxy ornithine carrying an acyl-moiety (100). Mycobactin has been shown to contain ϵ -N and its detailed structure has also been worked out (102). The amino acid sequence in ferrichrome has been studied (103) and a three dimensional structure has been proposed on the basis of several physico-chemical data (96,97). Ferrichrome A differs from ferrichrome in that it contains serine in addition to glycine and δ -N-hydroxyornithine, has less nitrogen and iron and the acyl moiety is replaced by β -methyl glutaconic acid (104). The hydroxamate moieties of ferrioxamines include 1-amino-5-hydroxyl amino pentane and 1-amino-4-hydroxylaminobutane. The organic acids present include acetic acid and succinic acid. Detailed structures have been proposed for the ferrioxamines as well as the ferrimycins (95).

Considerable evidence has accumulated in the case of ferrichrome, pointing to its important role in iron metabolism. It has been found that there exists a reciprocal relationship between ferrichrome and cytochrome C contents in the cells of Ustilago sphaerogena which is governed by the zinc status of the medium (57). Under conditions of zinc deficiency the cells contain little cytochrome C but yield relatively large amounts of ferrichrome With increase in the zinc status there is a drop in the ferrichrome content concomitant with the synthesis of cytochrome C. Later investigations have thrown light on the relation between ferrichrome and cytochrome contents, but the role of zinc is not clear. Ferrichrome has been found to be a growth factor for certain organisms like Arthrobacter terregens, Arthrobacter 8181 (105) and Arthrobacter JG 9. But a much large number of organisms have been found to produce growth factors of the ferrichrome type of at least answer for bound hydroxylamine. Detailed studies with Arthrobacter JG 9

revealed that it has an absolute requirement for ferrichrome to maintain normal growth and catalase activity even though inorganic iron may be provided in the medium. The ferrichrome requirement can be met by providing 100 times its amount of hemin (106). Ferrichrome - Fe^{59} been shown to get incorporated into catalase which is repressed by the addition of hemin in growing cultures of Arthrobacter JG 9 (107). It has been found that ferrimycin-A inhibits catalase synthesis in JG 9 when supplemented with ferrichrome but not provided with hemin. All these results have been interpreted to that ferrichrome is necessary for heme synthesis and ferrimycin A acts by blocking this step. Cell-free extracts of Rhodopseudomonas spheroides have been able to synthesise hemin, when incubated with an oxidizable substrate, protoporphyrin IX and iron as ferrichrome (108). It has been suggested that at least in microorganisms the sideramines can play an important part in the enzymatic incorporation of iron into

biological activity of the ferrichrome compounds in iron transport has a bearing on the affinity of these compounds for ferric and ferrous iron. Ferrichrome would bind ferric iron with a stability constant about 10 times that of EDTA; but it would bind ferrous iron if at all very weakly. Neilands has envisaged a mechanism for the release of iron from ferrichrome involving reduction and the iron-free moiety can bind ferric iron in turn to give the parent compound (57). The ferric iron coordinated as the trihydroxamate can be transported to or into the cell and donated to the iron enzymes by a mechanism which may involve one electron reduction. The reduction and release can take place at the site of incorporation into the iron containing enzymes and prosthetic groups (110).

Thus, the secretion of an iron-binding compound by an organism under iron deficient conditions has enormous survival value for the organism

in question. Neilands (99) in a discussion of the possible factors involved in this metabolic adjustment lists the following: (I) the biosynthesis of ferric complexing agents, normally competitively inhibited and maintained at a low level by the presence of a variable amount of a ferric chelate becomes a major metabolic activity of the cell.(II), the deficiency of iron creates a metabolic block, the latter being manifested by the appearance of iron-complexing products which usually require iron for their metabolism. For example, in Bacillus subtilis the accumulated itoic acid in iron deficiency, is rapidly removed on addition of iron, (III) the new substances produced in iron deficiency are intended to serve, either as such or as the ferric complex, as a by-pass for electron transport around the normal cytochrome system. The last mentioned possibility has been considered rather remote in view of the profound differences in the binding affinities of these compounds for ferrous and ferric iron.

It is evident that the isolation and the metabolic studies carried out on the siderochromes have far reaching effects in that the regulatory processes of the type operating in iron metabolism in microorganisms may as well be a phenomenon operating in higher plants and animals. It is well known that plants have an affinity for chelated elements and it has recently been shown that ferrioxamin B is absorbed and translocated in tomato plants from nutrient solution. The chelated iron is transported to the upper parts of the plants more rapidly than ionic iron (111). Studies are yet to be carried out in animal systems to detect the possible presence of ferrichrome type compounds. It has been considered probable that the non-availability of iron frequently observed in both acidic and alkaline soils may be due to the lack of a substantial microbial population and hence the absence of the ferrichrome class of compounds (110).

The sideromycins have a great antibiotic potential. Danomycin (112), a recently isolated antibiotic, has inhibitory properties towards Gram positive bacteria, and is an iron polypeptide related to the sideromycins.

A representative literature that exists on aspects of cobalt and nickel toxicities and iron deficiency in microorganisms has been reviewed. The knowledge gained has been substantial but still the diverse manifestations of a single metal deficiency or toxicity are baffling. It just emphazises the closely knit and integrated manner of operation of chemical processes of life, such that the lack of a small metabolite can upset the entire machinery. But each organism is endowed with a capacity to mend its own machinery and this results in the operation of survival mechanisms which in turn throw light on the normal regulatory processes which operate in the living cell. One such example is the elaboration of iron-binding compounds in iron deficiency. The formation of an organic iron chelate of this type in a normal cell probably represents a very early phase in the transport of iron to various iron dependent systems.

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CHAPTER I

ISOLATION, PROPERTIES AND DEGRADATIVE STUDIES ON AN IRON BINDING COMPOUND ISOLATED FROM COBALT-TOXIC CULTURES OF *NEUROSPORA CRASSA*

ISOLATION, AND DEGRADATIVE STUDIES ON AN IRON ISOLATED FROM COBALT-TOXIC CULTURES OF *NEUROSPORA*

Section A: Isolation and Properties.

It is evident from the literature review on trace metabolism that accumulation of metabolites is a feature when microorganisms are subjected to a trace metal deficiency such as that of iron. For example, organisms are known which accumulate both porphyrins and iron-binding compounds or either of the two, when grown under iron deficient conditions (1). These accumulated products may thus represent metabolites which require iron for their further metabolism. However, the metabolic potency of the ferrichrome type compounds as iron transporting agents has a particular significance in the sense that these compounds do not merely represent unmetabolized end products. They can bind any available iron and specifically transport it to key iron dependent systems and thus have a survival function to perform in the living cell.

Earlier studies in *Neurospora crassa* (2,3) have shown that a specific cobalt-iron antagonism is demonstrable in this organism and that cobalt toxicity leads to a conditioned iron deficiency. It has been of interest to find out whether *N. crassa* accumulates characteristic product or products when grown under conditions of iron deficiency and if so whether a similar phenomenon is detectable under cobalt toxic conditions as well. Preliminary studies have indicated that this organism does not accumulate porphyrins when grown under iron deficient or cobalt toxic conditions. However, when an iron salt such as ferric chloride is added

to the culture fluid of the organism grown under these conditions, it turns immediately red indicating the possible presence of an iron-binding compound in the culture fluid. Subsequently, this has been traced to the presence of a new iron-binding substance belonging to the siderochrome class of compounds. The isolation and some of the properties of this new iron-binding compound are reported in this section.

EXPERIMENTAL

Materials.

Analytical grade salts were used in constituting the basal medium of the organism. Metal free water prepared by passing glass distilled water through ion-exchange resin was used for preparing the medium. Benzyl alcohol used for extracting the iron-binding compound was laboratory grade reagent (BDH). Paper chromatography and electrophoresis were carried out on Whatman No. 1 filter paper.

Organism.

A wild strain of *Neurospora crassa* Em 5297a was used in these studies. It was maintained by weekly subcultures on agar slants containing the constituents of the basal medium (given below) along with 0.1% each of malt and yeast extracts. A spore suspension from a 7 day old culture in sterile glass distilled water adjusted to nearly 90% transmission in a photoelectric colorimeter was used for inoculation.

Basal medium and growth conditions

The basal medium contained: (g/100 ml) glucose-2; KH_2PO_4 - 0.3; NH_4NO_3 -0.2; ammonium tartrate - 0.1; $MgSO_4 \cdot 7H_2O$ - 0.05; NaCl - 0.01; $CaCl_2$ - 0.01; Trace elements included were (µg/100 ml): zinc -20;

manganese - 20; copper - 8; iron - 2; molybdenum -2. Biotin was added to give a final concentration of 0.5 µg/100 ml. Cobalt as $CoCl_2.6H_2O$ was included at 800 µg/10 ml basal medium to produce around 50% growth inhibition at the end of 72 hours.

N. crassa was grown in 50 ml pyrex conical flasks containing 10 ml of the basal medium adjusted to pH 4.8 - 5.0, for 72 hr at 30 ± 1° in stationary cultures.

Isolation of the iron-binding compound.

The iron-binding compound (X) was isolated as the iron-complex (XFe) from the culture filtrate on a preparative scale, when the organism was grown under cobalt toxic conditions.

N. crassa was grown in a large number of pyrex conical flasks containing 800 µg cobalt/10 ml basal medium for 72 hr. in stationary culture. At the end of the growth period the culture fluids were pooled. To a litre of the culture fluid a solution of $FeCl_3.5H_2O$ was added drop by drop with stirring. The solution turned immediately red followed by copious precipitation. Enough iron salt was added so that the red supernatant obtained after centrifugation did not show any increase in absorbancy at 440 mµ with further additions of the iron salt. The clear red supernatant was then saturated with $(NH_4)_2SO_4$ and left at 4° overnight. It was filtered and then extracted thrice with benzyl alcohol using 50 ml each time. The red color was quantitatively extracted into the organic phase and the pooled organic layer was then washed four times with equal volumes of metal-free water. The water wash was discarded. The benzyl alcohol layer was shaken with an ether:water (5:1) mixture when the red color was extracted into the aqueous phase. The aqueous layer was drawn

and collected. The red color was completely extracted into the aqueous phase with further additions of water to the ether-benzyl alcohol solution. The pooled aqueous layers were then extracted twice with ether using 50 ml each time, to remove traces of benzyl alcohol and then lyophilized. The concentrate was fed on to a cellulose column (30 cm x 2.5 cm) and eluted with 50% methanol when the single red band moving on the column was collected and lyophilized again.

Yield: The lyophilized material weighed mg of the culture fluid.

iron-deficient media.

An iron-binding compound was also isolated from the culture fluid when the organism was grown under conditions of straight iron deficiency. The isolation procedure employed was the same as described

The iron-deficient medium was prepared by constituting the omitting iron, in metal-free water. Glucose (A.R) was rendered by shaking the sugar solution with Dowex-50 H^+, resin and then to free it of the sugar. KH_2PO_4, ammonium tartrate ammonium nitrate were freed of metals by shaking repeatedly with 8-hydroxyquinoline in chloroform until the chloroform layer was no longer colored blue. Excess 8-hydroxyquinoline was removed by repeated extractions the chloroform was removed by

constituted was deficient in iron and permitted 50% of the mycelial growth (in terms of dry weight) at the end of 72 hr. as compared to that obtained in a normal medium containing optimal levels of iron.

Hydrolysis of XFe.

10 mg of XFe was hydrolysed with 2 ml of 6 N HCl in evacuated sealed tubes for 22 hr. at 105°. The hydrolysate was evaporated in vacuum to dryness. To the residue a small amount of water was added and again evaporated. This process was repeated thrice to remove the hydrochloric acid and finally the residue was dissolved in 10% isopropanol and aliquots were spotted on two dimensional chromatograms for amino acid analyses.

chromotographic electrophoretic

The homogeneity and the chromatographic mobility of XFe were tested on whatman no. 1 filter sheets in ascending runs using butanol: acetic acid:water (4:1:1) and methanol:water (1:1) as the solvent systems. Ferrichrome and ferrichrome A were also spotted for comparison. XFe hydrolysate was analysed for amino acids by two dimensional chromatography

the first descending run and

phenol:ethanol:water (3:1:1) containing 0.05% 8-hydroxyquinoline for the

ascending run in an ammonia atmosphere. Hydroxylamine in XFe acid hydrolysate was detected in one dimensional ascending chromatograms using

(different proportions) as the solvent system.

of XFe was checked electrophoretically and its mobility compared with that of ferrichrome and ferrichrome A on whatman No. 1 filter strips at three different pHs. The buffers used were pyridine: acetic acid:water (1:10:190 - pH 3.6), pyridine:acetic acid:water (20:2:178 - pH 6.2) and veronal:HCl (50 ml of 0.2 M sodium barbital + 2.5 ml of 0.2 M HCl, diluted to a total of 200 ml - pH 9.0)

Spray reagents.

Ninhydrin was used for detecting aminoacids on paper. A 0.3% solution in acetone was used. Specific spray reagents were used to confirm the presence of certain amino acids (4). A 0.25% solution of O-pthaloldehyde in acetone was used to detect glycine. The presence of ornithine was confirmed by spraying the chromatogram with a 0.2% solution of vanillin followed by 1% alcoholic KOH. Ornithine gave a red colour on heating. Serine and threonine were detected by periodate spray followed by Nessler's

A 1% solution of triphenyl tetrazolium chloride in 1N NaOH was

to

RESULTS

Solubility.

The isolated iron-complex (XFe) is freely soluble in water and cold methanol. It is insoluble in lipid solvents like ether, acetone and chloroform. Ferrichrome and ferrichrome A are sparingly soluble in cold methanol and cold water respectively. The ferrichromes are however insoluble in lipid solvents (1).

omatographic and electrophoretic behaviour

XFe gives a homogeneous spot on paper when developed with the butanol:acetic acid:water (4:1:1) and methanol:water (1:1). No reaction is obtained at the XFe spot or elsewhere when the developed chromatograms are sprayed with ninhydrin, anilinephthalate or bromocresol green. The XFe spot appears dark when viewed under the ultra violet, but the chromatograms do not reveal any other quenching or fluorescent spots. Table I indicates the Rf values of XFe in the two solvent systems mentioned as well as those of ferrichrome and ferrichrome A. XFe has a

mobility as compared to ferrichrome or

Paper electrophoresis of XFe along with ferrichrome indicates that the two have identical mobilities at all the pHs examined and give homogeneous spots. The compounds have cathodic mobilities at all the pHs examined. At pH 6.2, XFe and ferrichrome have distinctly different chromatographic mobilities as compared to ferrichrome A (Fig. I).

Hydrolytic Products.

6N HCl hydrolysis of XFe reveals the presence of atleast 9 ninhydrin positive spots when examined on two dimensional chromatograms employing butanol:acetic acid: water (30:6:14) and phenol:ethanol:water (3:1:1) as the developing solvents. The amino acids have been identified by their characterestic Rf values. Ornithine, glutamate, serine, glycine and alanine correspond to the major spots. Minor spots of aspartate and threonine can also be detected. The presence of ornithine, glycine, serine and threonine have been further confirmed by their characterestic color reactions on the paper chromatograms.

Hydroxylamine has been detected in the hydrolysate by tetrazolium spray on one dimensional chromatograms developed by the use of ethanol:HCl solvent. The presence of hydroxyl amine is revealed by the characterestic red color observed when sprayed with the reagent and R_f falues obtained in the solvent system employed (Table II)

Spectrum.

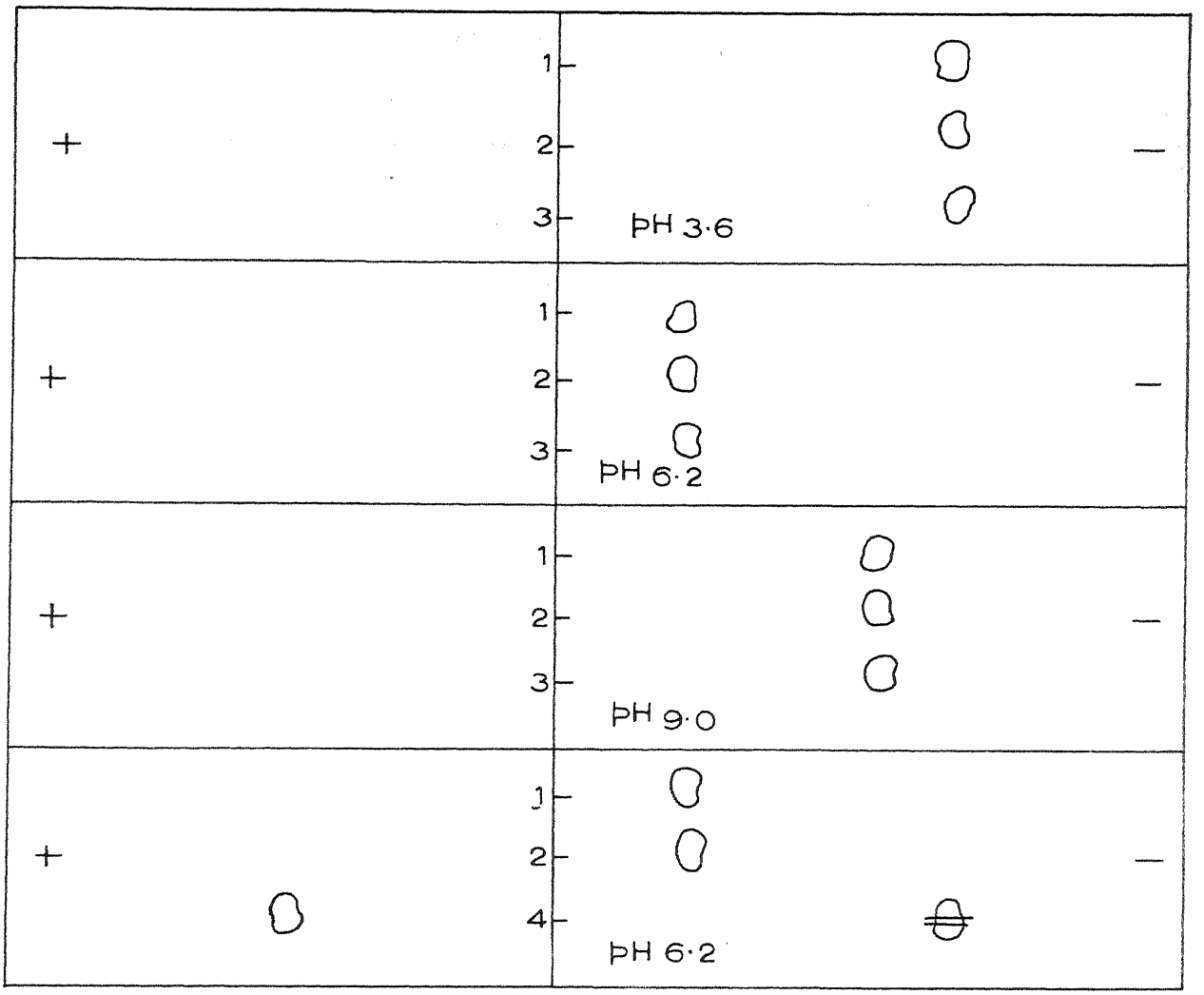
XFe has a broad absorption maximum at 440 mμ though it has only end absorption in the ultraviolet (Fig. II).

Affinity for iron.

The compound has been found to hold iron with great affinity. When a sample of XFe containing 5 μg of iron in aqueous solution is shaken

Fig.I

Electrophoretic mobilities of Ferrichrome, Ferrichrome A and the iron-complexes isolated under conditions of cobalt toxicity and iron deficiency.



1. Iron-complex isolated under cobalt-to
2. Ferrichrome. 3. Iron-complex isolated under i deficient conditions. 4. Ferrichrome A.

The figure is drawn to scale.
carried out at 800 V for 2 hr

Table I

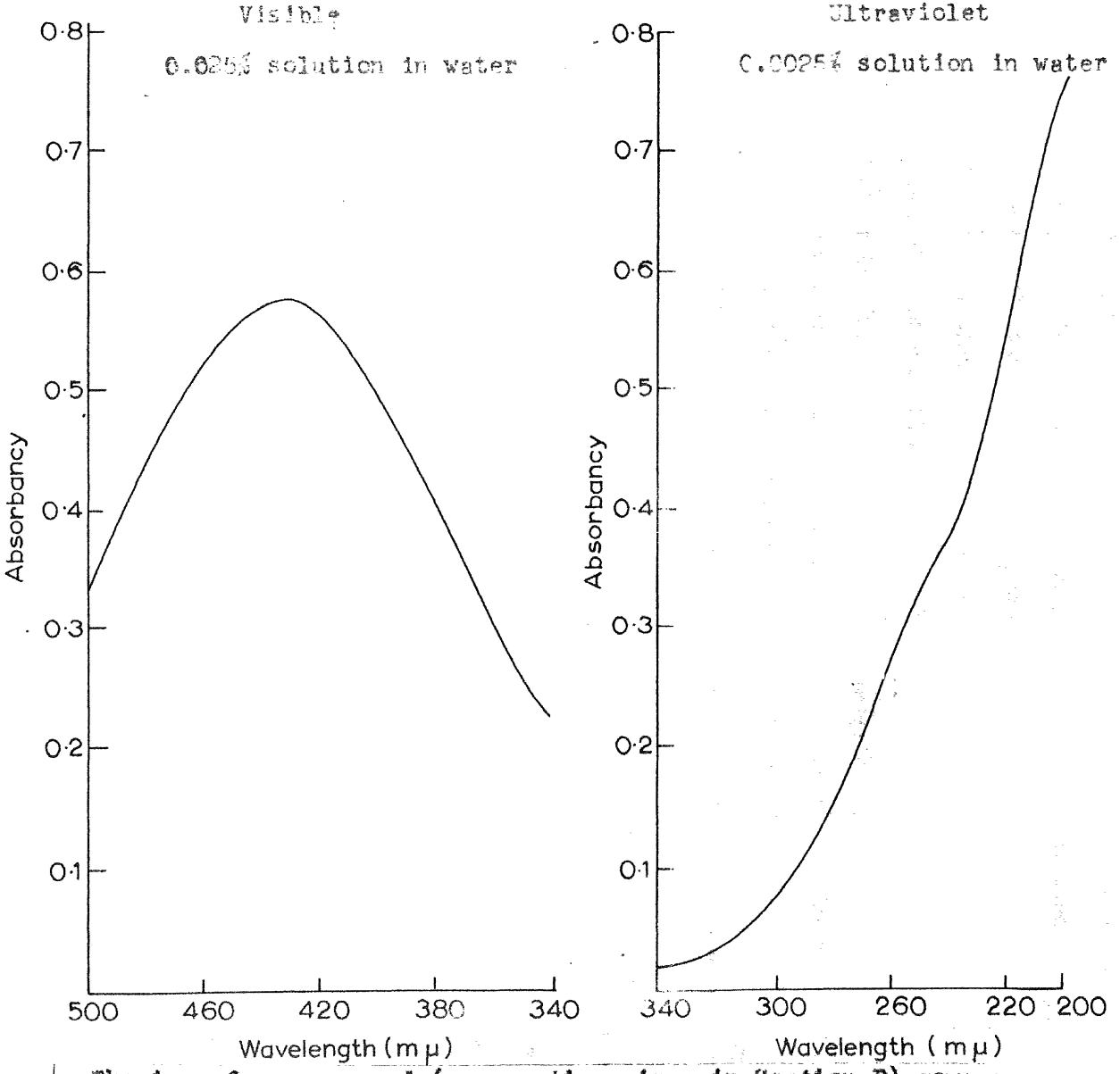
R_f values of ferrichrome, ferrichrome A and XFe

Solvent system	R_f values of iron-complexes			
	Ferrichrome	Ferrichrome A	XFe isolated under	
			Cobalt toxicity	Iron deficiency
Butanol:acetic acid: water (4:1:1 -ascending)	0.38	0.44	0.56	0.56
Methanol : water (1:1 - ascending)	0.79	0.72	0.88	0.88

Table

Rf values of the tetrazolium positive material in the XFe acid hydrolysate

Solvent system (95% ethanol: 6N HCl)	Rf values	
	Tetrazolium positive material	Hydroxylamine
80:20	0.37	0.37
60:40	0.51	0.51
40:60	0.67	0.67
20:80	0.82	0.82



The iron-free compound (preparation given in Section B) gave a similar uv spectra as that of XFe and did not reveal any characterestic peaks.

with 40 µg of 8-hydroxyquinoline in chloroform (at a molar ratio of 1:3 for iron:8-hydroxyquinoline) for 4 hr., it has been found that the loss of iron from the parent compound is only 4.3%. Details of iron estimation procedure are given in Section B. Addition of $\alpha\alpha'$-dipyridyl or orthophenanthroline to a dilute aqueous solution of XFe does not produce the characterestic red color of the ferrous iron-complex. However, when the dilute aqueous solution of XFe (0.025% w/v) is reduced with 25 mg of sodium dithionite or an equal amount of ascorbic acid, subsequent addition of $\alpha\alpha'$-dipyridyl or orthophenanthroline gives rise to the characterestic red color, indicating the presence of ferrous iron. Dithionite reduction of a dilute solution of XFe results in decolorisation and the color is regained on subsequent aeration. This color is not regained when the reduction is carried out in presence of cyanide. Presumably, XFe in the natural state contains ferric iron, which it binds strongly. It binds ferrous iron, if at all, very

DISCUSSION

The iron-binding compounds isolated under cobalt-toxic and straight iron-deficient conditions have identical chromatographic and electrophoretic mobilities (Table I and Fig. I). When the samples are hydrolysed with acid under identical conditions, the qualitative amino patterns obtained on two dimensional chromatograms are similar. It appears that the same iron-binding compound is excreted by the organism when grown under cobalt-toxic or iron-deficient conditions.

XFe appears different from the ferrichromes on the basis of solubility characterestics and chromatographic mobility. Identical electrophoretic mobilities of XFe and ferrichrome at different pHs indicate that XFe is also a neutral compound like ferrichrome. Ferrichrome A has been shown to be unlike ferrichrome (6).

It has been pointed out (Prof. J.B. Neilands, Personal communication) that δ-N-hydroxyornithine, a constituent amino acid of the ferrichromes undergoes a baffling series of disproportionation reactions on acid hydrolysis in presence of iron giving rise to several ninhydrin positive spots. Subsequent[6] studies on XFe have revealed this to be the case although the amino acid analyses of the iron-free compound shows it to be different from the ferrichromes. These aspects are discussed in Section B.

The presence of a strong binding affinity for ferric but not for ferrous iron, the detection of a broad absorption maximum at 440 mμ in the spectrum of XFe and hydroxylamine in the acid hydrolysate of XFe, indicate that the N. grassa compound is related to the ferrichromes and the other members of the siderochrome (7) class of compounds. Degradative studies on XFe have been carried out in order to have an insight into its structure and these results are presented in Section B.

SUMMARY

1. A new iron-binding compound has been isolated as the iron-complex (XFe) from cobalt-toxic cultures of N. grassa

2. An iron-binding compound is also obtained when this organism is grown under conditions of straight iron deficiency. It appears that the same compound is secreted under conditions of cobalt toxicity and iron deficiency.

3. Preliminary studies indicate XFe to belong to the siderochrome class of compounds but its properties show it to be different from the ferrichromes.

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Section B. Degradative studies on the iron-binding compound isolated from cobalt-toxic cultures of *Neurospora crassa*

Preliminary studies have indicated that XFe has properties closely related to the siderochromes, the term siderochromes(1) being referred to the class of iron-binding or iron-containing compounds with a polyhydroxamate structure. The results described in this section deal with the studies on XFe, aimed to reveal the similarities and differences in the types and amounts of the degradation products obtained from this new compound with those reported for the already known siderochromes.

EXPERIMENTAL

Isolation of XFe.

XFe was isolated from cobalt-toxic cultures of *Neurospora crassa* as described in section A. The product obtained was homogeneous on paper chromatograms in several solvent

Estimation of iron

The iron content of XFe was determined using O-phenanthroline(2), after wet ashing the sample with nitric-sulphuric acid mixture. 5 mg of XFe was digested with an acid mixture containing 2 ml of nitric and 0.5 ml of sulphuric acids. Evaporation was continued till white fumes of sulphuric acid were liberated. The digest was cooled, 1 ml of water added and again evaporated to white fumes. The process was repeated thrice. The clear digest was finally made upto a known volume with water and aliquots containing 5 -20 µg iron were used for color development.

A 2 ml aliquot was neutralized with aqueous ammonia to pH

by the addition of 1 ml of sodium acetate-acetic

solution (10 gm of sodium acetate, 40 ml of glacial acetic acid and 200 mg of hydroquinone made up to 100 ml with water). 0.2 ml of a 5% solution of O-phenanthroline in water was added and the volume made up to 5 ml. The red color developed after 30 min. was measured in a Klett-Summerson colorimeter with filter No. 49.

ammonium sulphate (A.R.) subjected to the same ashing procedure was used as the standard iron source. Suitable digestion and reagent blanks were employed.

of XFe and the compound.

Hydrolysis of XFe was carried out with 6N HCl. Degradative studies on the iron-free compound were carried out using periodate, 6N HCl and 2N NaOH.

of

Three different methods were examined for the removal of iron from XFe. These procedures involved treatment of the compound with (a) acid (b) alkali and (c) chromatography on ion-exchange resin.

A 10 mg quantity of XFe was dissolved in 1 ml of water and 1 ml of N. KOH was added. The solution was allowed to stand for an hour at 4° and then centrifuged. The colorless supernatant was immediately adjusted to pH 5.0 with N. HCl and then saturated with ammonium sulphate. It was extracted three times with benzyl alcohol using 5 ml for each extraction.

layer was carefully removed after centrifugation and was

with ether:water (5:1) mixture. The water layer was drawn and collected. Further extractions with small quantities of water were carried until the aqueous phase when treated with a solution of ferric chloride did not give the characterestic red color of the iron-complex. The aqueous layers were pooled, washed thrice with ether and then lyophilized. The material was dried over P_2O_5 in vacuum for 24 hr. and

The product obtained was faint yellow in color but did not contain any iron that could be detected by O-phenanthroline method. The alkali method

works successfully for iron removal from ferrichrome (3) has been

to yield a colorless product. It is therefore possible that slight yellow color of the iron-free compound from XFe was due to some unidentified organic component present in it.

Resin method.

The chromatographic behaviour of XFe was studied using two cation exchange resins and an anion exchange resin.

Chromatography on cation exchange resins.

A 10 mg quantity of XFe in 1 ml of water was placed on an Amberlite IRC - 120 (H^+) column (10 cm x 1 cm) and eluted with water. The break through fluid gave an intense red color when tested with ferric chloride solution. The effluent was collected till the test solutions did not give any color with ferricchloride and lyophilized. The product obtained was a pale yellow highly hygroscopic solid. This was taken in 2 ml of water and was stored at 4°.

of 0.6 ml of 0.1 N periodic acid. The acidic solution was extracted with ether five times, using 4 ml each time, after adding a drop of ethylene ether was evaporated and the residue was dissolved in water and used for spectral and paper chromatographic studies.

Procedure for acid hydrolysis.

The iron-free compounds obtained by alkali or resin treatment from 10 mg quantities of XFe were hydrolysed with 2 ml of redistilled 6N HCl for 22 hr in pyrex glass tubes sealed in vacuum, at 105°. Due precaution was taken to see that the air in the tubes was completely removed before they were sealed. The hydrolysate was evaporated to dryness in vacuum. A small amount of water was added to the residue and was carried to dryness again. The process was repeated thrice and the final residue obtained was used for amino acid analysis.

A sample of XFe, 10 mg, was subjected to alkali treatment to remove the iron as described earlier and the solution thus obtained was to 2N alkali concentration. This solution was taken in a pyrex test evacuated , sealed and heated at 105° for 22 hr. The hydrolysate was neutralized with HCl and an aliquot of this solution was used for amino acid analysis.

hydrogenation of the acid hydrolysate.

The product obtained by acid hydrolysis of the iron-free compound obtained from 1.5 mg of XFe, was dissolved in 2 ml of water and was reduced by passing a stream of hydrogen, in presence of 10 mg of PtO_2, for an hour.

The catalyst was removed by centrifugation and was washed several times with 1N. HCl. The supernatants were pooled, concentrated in vacuum and used for amino acid analysis.

analysis.

Qualitative detection of amino acids was carried out by paper chromatography and paper electrophoresis.

Desalting.

For paper chromatographic and electrophoretic analyses, the alkali hydrolysates were desalted by extracting the residue repeatedly with acetone containing 5% of 6N. HCl (4). The acid-acetone soluble material was dried in vacuum and the residue was dissolved in water. Aliquots were then used for chromatography and electrophoresis on paper.

Paper Chromatography.

The aminoacids in the hydrolysates were identified by circular paper chromatography on Whatman No. 1 filter sheets using butanol:acetic acid: water (4:1:1) as the solvent system. This system was not found to be suitable for the separation of ornithine and lysine. Where separation of these two aminoacids was needed the solvent system (5) used was ethylene-glycol monomethyl ether:propionic acid: 5N NaCl: water (350:75:26:49). The ether extractable material obtained after periodate treatment of the iron-free compound was chromatographed on Whatman No. 1 paper using butanol: formic acid:water (100:15:150) in the ascending system.

Paper electrophoresis

The iron-free compound acid hydrolysate as well as the product obtained after reducing the acid hydrolysate, were subjected to electro - phoresis on Whatman No.1 paper for 2 hr at 600 volts using pyridine:acetic

citric acid: water (40:30:12:930), pH 4.9. The amino acids were detected on paper by the use of a 0.3% solution of ninhydrin in acetone. Tetrazolium spray (described in Section A) was used to detect the hydroxamate function.

Quantitative analysis of amino acids with the automatic aminoacid analyser

The quantitative estimation of the aminoacids obtained under different hydrolytic conditions was carried out in an automatic amino acid analyser of the type developed by Spackman, Stein and Moore (6) and installed in this laboratory from the basic components of a Beckman model 120B analyser.

The unit consisted of reservoirs containing buffer solutions and ninhydrin reagent, pumps and columns of ion-exchange resin. The resin used for chromatography was sulphonated styrene 8% divinyl benzene copolymer prepared at the National Chemical Laboratory, Poona. This was subsequently processed and fractionated into 4 different grades of uniform particle sizes for the operation of the different columns. Two types of columns each (operating under normal and accelerated effluent flow rates) were generally used for the separation of acidic and neutral amino acids and of basic amino acids.

In the present study, the acidic and neutral amino acids were separated on a 0.9 x 60 cm column packed with resin particles ranging from 15 -20 microns in size and operated at an accelerated effluent flow rate. For complete separation of the amino acids normally present in a protein hydrolysate, this accelerated run required elution with 0.2 N citrate buffer (pH 3.28) at a rate of 40 ml per hr for 3 hr 30 min followed by elution with 0.2 N citrate buffer (pH 4.25) for another 4 hours.

The basic amino acids were separated on a 0.9 x 15 cm column containing the resin of particle size 25 - 30 microns. This column was

developed at the normal eluant rate of 30 ml per hr. using 0.35 N citrate buffer (pH 5.28).

All the runs were carried out at 50°. The ninhydrin flow rate for the normal run was 15 ml per hr. whereas the flow rate for the accelerated run was 20 ml per hour.

The other accessories of the unit included the mixing manifold, the reaction coil of Tefflon tubing(100' in length) maintained at 100°, the absorption meter and the recorder.

The absorption meter consisted of 3 cells and the accessory components, 2 of which were used for measurement of absorption at 570 mμ and the other for absorption at 440 mμ. The absorptions at 570 mμ were recorded at two concentrations in the automatic recorder to facilitate estimation of aminoacids in the range 0.2 - 3.0 μmoles with an accuracy of more than 95%. The recorder was operated at a chart speed of 62.5 mm per hr for a normal run and at 125 mm per hr for an accelerated run.

The color value and the peak position for each amino acid present in a standard mixture was calculated from a chromatographic run of the amino acids under normal and accelerated conditions. The color value for ornithine was calculated from a run of this amino acid alone. It was observed that the peak position for ornithine coincided with that of lysine and in all estimations of ornithine using the automatic amino acid analyser sufficient care was taken to verify lysine contamination by running simultaneous circular paper chromatograms as described earlier.

RESULTS

The iron content of a chromatographically and electrophoretically

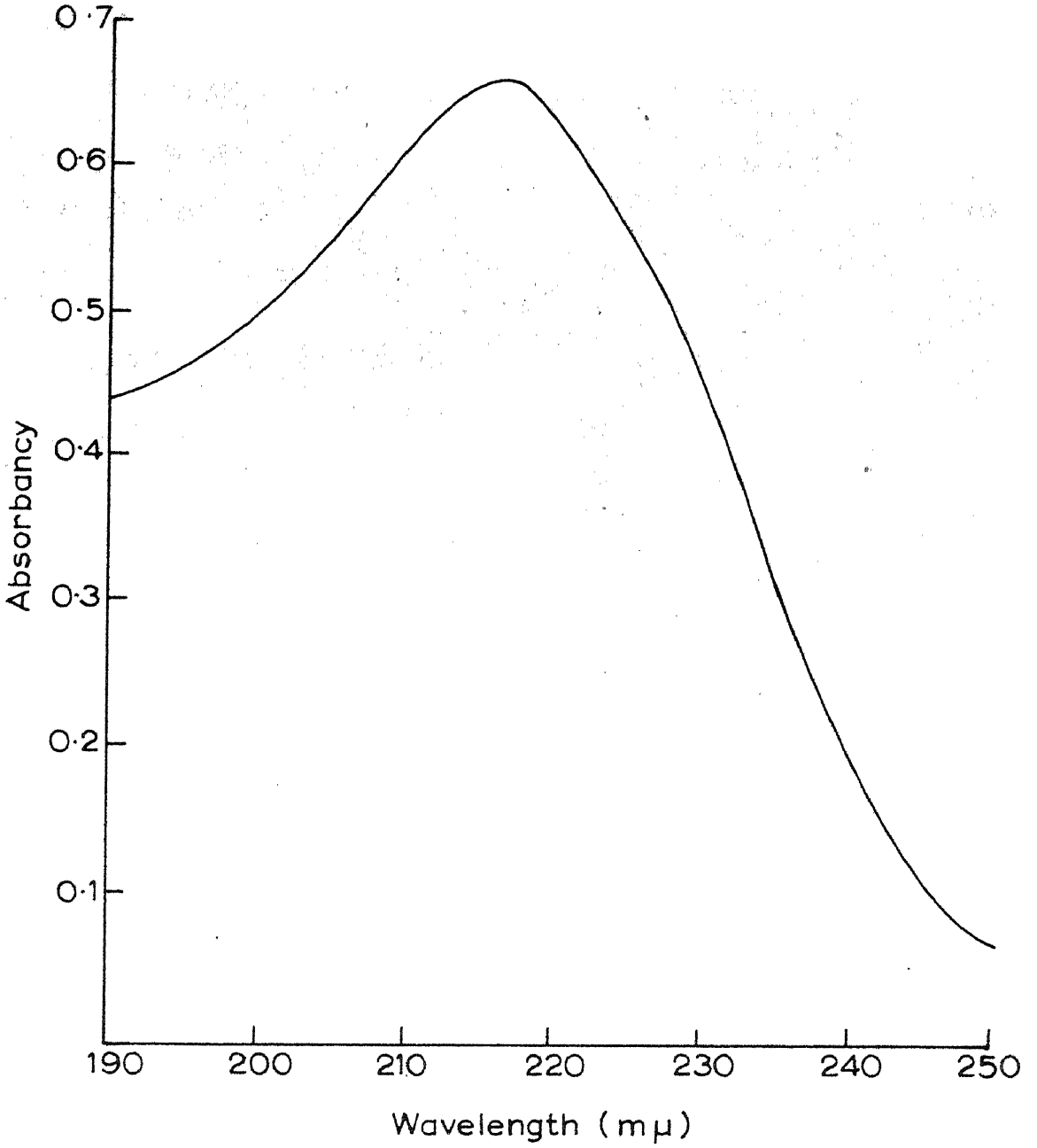
pure sample of XFe, as determined by the orthophenanthroline method described in the experimental section has been found to be 4.48%. With the assumption that 1 mole of XFe contains 1 mole of iron, the compound can be assigned a molecular weight of 1228.

The alkali as well as the resin methods have been found to remove iron from XFe effectively. In a typical experiment, a 10 mg quantity of XFe after removal of the metal by alkali treatment has not been found to contain any chemically detectable iron in it. In another experiment, XFe^{59} (Prepared by adding $Fe^{59}Cl_3$ directly to the cobalt-toxic culture fluid) containing 13420 cpm/10 mg of the compound is passed through a Dowex 50 (H^+) resin column. The iron-free compound isolated has not been found to contain any measurable radioactivity.

Periodate treatment of the iron-free compound prepared by alkali method gives a residue which has been found to be acidic in nature. This residue decolorises both alkaline permanganate and bromine water. These properties are indicative of the unsaturated nature of the acidic residue. An aqueous solution of the unsaturated acid has an ultra-violet absorption spectrum with a maximum absorption in the range 214-217 mμ (Fig. I). This material when chromatographed on paper developed with butanol:formic acid; water solvent moves with an Rf of 0.84. The material has been detected on paper as a red spot when sprayed with dichlorophenol indophenol indicator. Emery and Neilands(3) have found that ferrichrome A contains an acid moiety attached to the hydroxamate function which is unsaturated. It has an absorption maximum at 217 mμ in 0.1 N HCl indicating the presence of an alkyl substituted double bond and has an Rf value of 0.87 in butanol:formic acid:water. On the basis of further studies including infra-red spectrum, reduction to -

Fig.I

Ultra-violet absorption spectrum of the acid material extracted with ether after periodate treatment of the iron-free compound prepared from xFe.



The spectrum was taken in aqueous solution. The experimental details are given in text.

β -methyl glutamic acid and gas chromatographic behaviour the authors have concluded that the acid material is β -methyl glutaconic acid. The properties of the unsaturated acid obtained from the iron-free compound of N.crassa indicate it to be β-methyl glutaconic acid or a compound closely related to it. It can be seen from Fig. I that the unsaturated acid obtained from 6.5 mg of XFe taken in 84 ml of water has an optical density of 0.656 at 214 - 217 mμ. Based on the E_{max} value for β-methyl glutaconic acid as 10,600 (3) and the molecular weight for XFe as 1228, the concentration of the unsaturated acid is 1 mole per mole of the iron-complex.

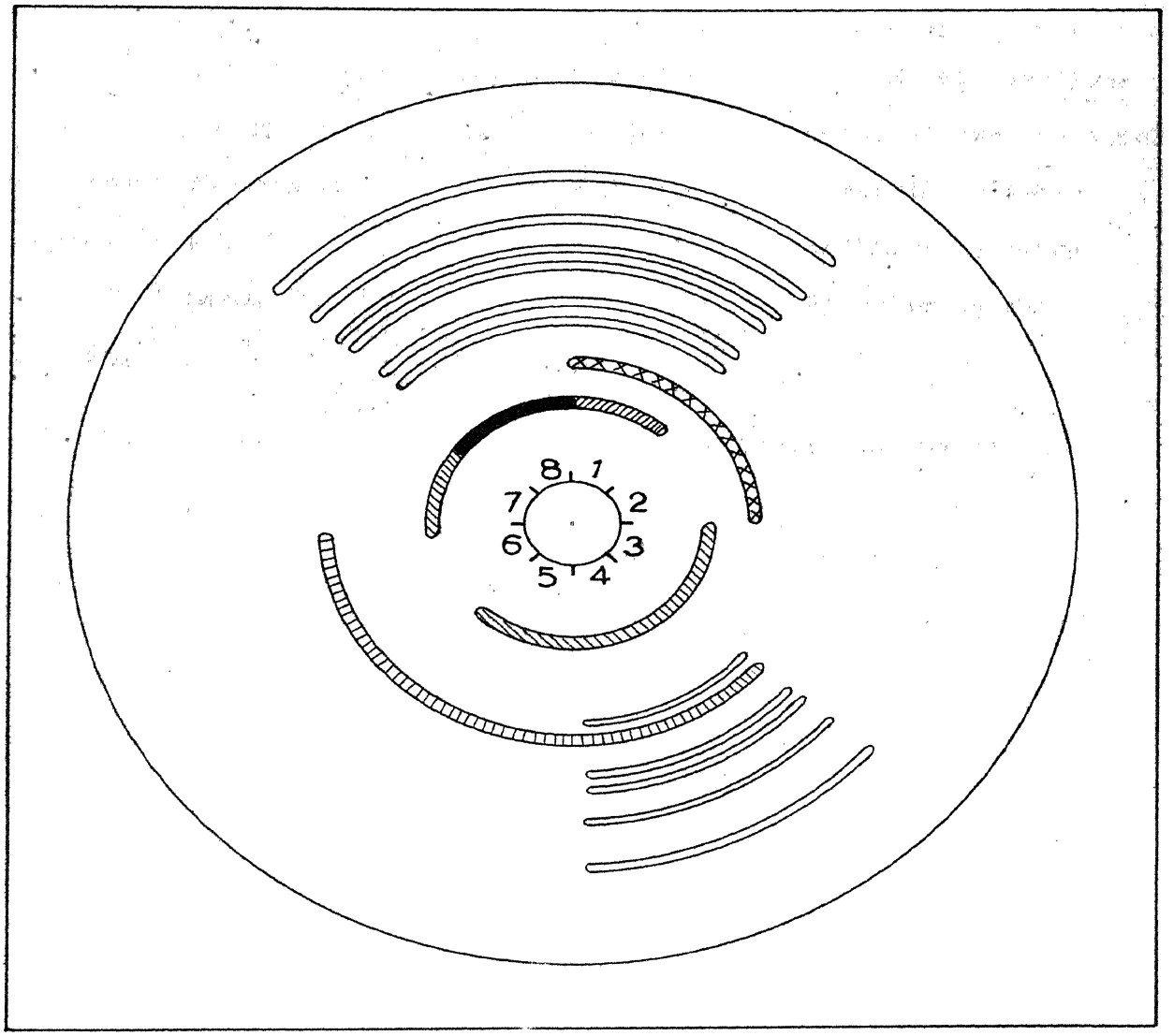
Characterization and estimation of the aminoacid components of XFe

It has been reported (Section A) that acid hydrolysis of XFe gives a complex mixture of amino acids and the possibility has been indicated that these could have arisen as a result of disproportionation reaction of the hydroxamate function in presence of iron. In order to arrive at some tentative conclusions as to the types and amounts of amino acid residues and functional groups present in XFe, analysis of the degradation products obtained from the iron-free compound under controlled hydrolytic conditions have been carried out.

The results obtained from a study of the circular paper chromatographic behaviour of the acid and alkali hydrolysates of the iron-free compound (prepared by alkali treatment) are depicted in fig.II. In this chromatogram are also included for comparison the catalytic reduction product of the acid hydrolysate, δ -N-hydroxy ornithine, ornithine, glutamic acid and lysine.

From fig.II it is clear that authentic samples of ornithine, δ -N-hydroxy ornithine and glutamic acid are distinctly separated in this system. Ornithine and lysine do not separate. The band in position

Circular paper chromatographic pattern of the amino acids present in the acid hydrolysate, reduced acid hydrolysate and alkali hydrolysate of the iron-free compound prepared from xFe.



The fig. represents a chromatogram run in butanol:acetic acid:water (4:1:1) and is drawn according to scale. The bands were developed with ninhydrin reagent.
1. Acid hydrolysate of the iron-free compound. 2. δ-N-hydroxy ornithine. 3. Ornithine. 4. Alkali hydrolysate of the iron-free compound. 5. Alkali hydrolysate of δ-N-hydroxy ornithine. 6. Glutamic acid. 7. Lysine.
8. Reduced acid hydrolysate of the iron-free compound.

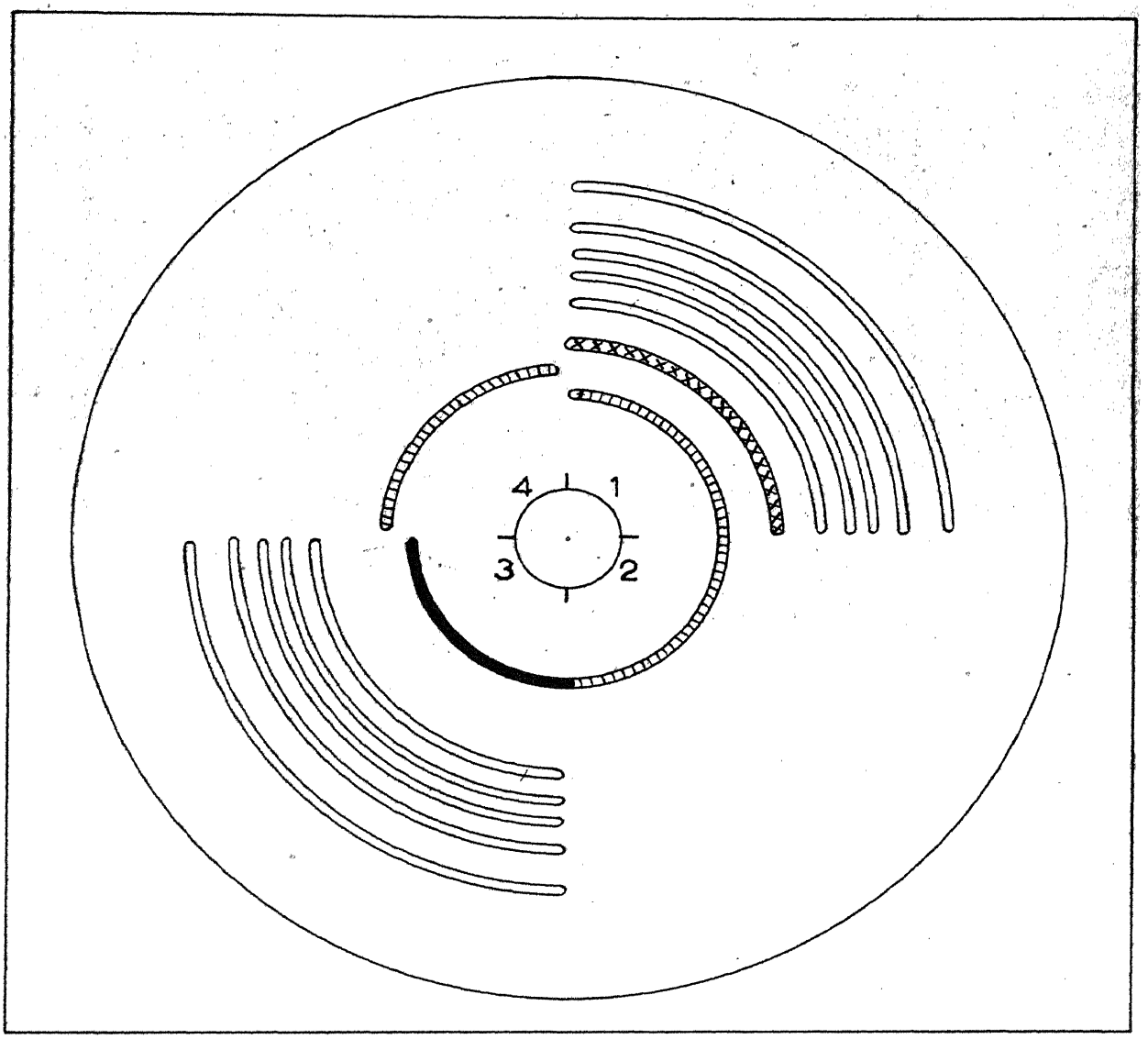
Intense band
Less intense band
Faint band
Ninhydrin and tetrazolium positive band

corresponding to that of δ-N-hydroxy ornithine gives the characterestic red color when sprayed with the tetrazolium reagent.

The acid hydrolysate of the iron-free compound contains mainly two distinct ninhydrin positive compounds chromatographing in the positions of δ-N-hydroxy ornithine and ornithine (or lysine). Traces of certain other ninhydrin positive compounds have also been detected. That the dibasic amino acid is not lysine has been verified by paper chromatography using ethylene glycol monomethyl ether:propionic acid:5 N NaCl: water as the solvent (fig. III).

The band corresponding to δ-N-hydroxy ornithine in the acid hydrolysate has been found to be tetrazolium positive. When the acid hydrolysate is reduced and chromatographed the band corresponding to δ-N-hydroxy ornithine disappears completely and the band corresponding to ornithine more intense (fig.III). Further, the electrophoretic behaviour of the products of acid hydrolysate and those of the reduced hydrolysate been studied and are depicted in fig. IV. It can be seen that the fastest moving spot with the cathodic migration has the same mobility as that of an authentic sample of ornithine. The slower moving spot is both ninhydrin and tetrazolium positive and has the same mobility as that of δ-N-hydroxy ornithine. The spot near the origin represents the position of neutral amino acids and probably represents the mixture of all the traces of degradation products. It is clear from the electrophoretic mobility of the products of the reduced hydrolysate, that the spot corresponding to δ-N-hydroxy ornithine has completely disappeared with a concomitant intensification of the ornithine spot. These studies clearly indicate that the iron-free compound on acid hydrolysis yields only two amino acid components in significant

Circular paper chromatographic pattern of the amino acids present in the iron-free compound acid hydrolysate, reduced acid hydrolysate, ornithine and lysine.



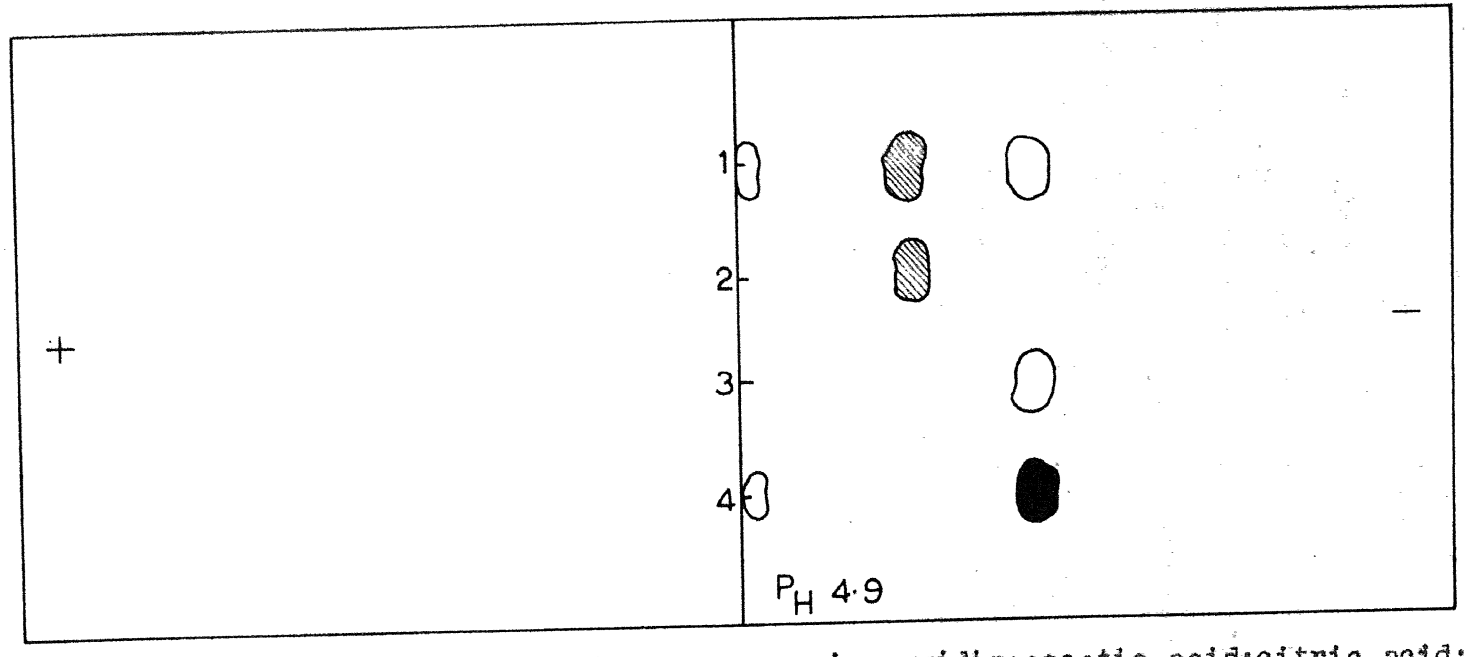
The fig. represents a chromatogram run in ethylene glycol monomethyl ether:propionic acid:5N Nacl:water (350:75:26:49) and is drawn according to scale. The bands were developed with ninhydrin reagent.

1. Acid hydrolysate of the iron-free compound. 2. Ornithine. 3. Reduced acid hydrolysate of the iron-free compound. 4. Lysine.

Intense band
Less intense band
Faint band
Ninhydrin and tetrazolium positive band

Fig.IV

Electrophoretic mobilities of the amino acids present in the iron-free compound acid hydrolysate, reduced acid hydrolysate, δ - N - hydroxy ornithine and ornithine.



The fig. represents an electrophorogram run in pyridine:acetic acid:citric acid: water (40:30:12:930-pH 4.9) at 600 V for 2 hr. and is drawn to scale. The spots were developed with ninhydrin spray.

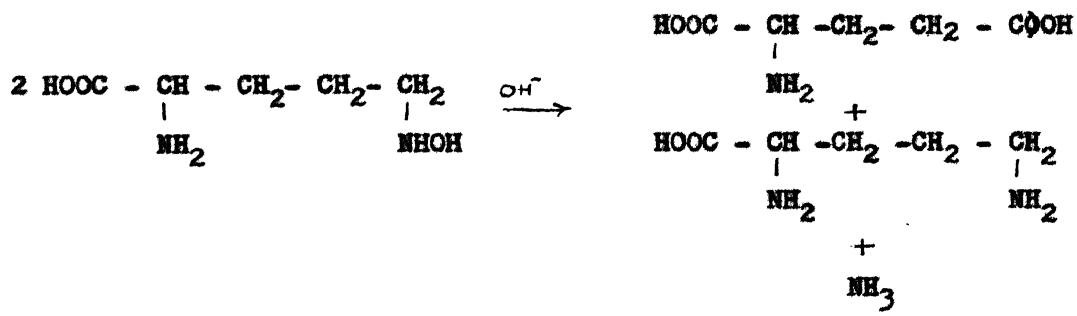
1. Acid hydrolysate of the iron-free compound. 2. δ - N- hydroxy ornithine. 3. Ornithine. 4. Reduced acid hydrolysate of the iron-free compound.

● Intense spot
○ Less intense spot
◍ Ninhydrin and tetrazolium positive spot

amounts namely ornithine and δ-N-hydroxy ornithine.

The alkali hydrolysate of the iron-free compound has also been found to give two ninhydrin positive components (fig. II) in significant amounts. Of these, one has the same chromatographic mobility as that of ornithine and the other with that of glutamic acid. The presence of ornithine has been confirmed by separate chromatography in the solvent system described earlier. Further, the presence of all these amino acids have been confirmed in subsequent studies using the automatic amino acid analyser.

The detection of glutamic acid in the alkali hydrolysate suggests the possibility that it may be present as such or as its γ-hydroxamate in the N. crassa iron-binding compound. However, the fact that the acid hydrolysate of the iron-free compound does not contain significant amounts of glutamic acid indicates that this may not be the case. So attention has been given to the possibility that δ-N-hydroxy ornithine may give rise to ornithine and glutamic acid by the following sequence of reactions.



It is with this in view that a study of the alkali hydrolysis of δ-N-hydroxy-ornithine itself has been carried out. It can be seen from fig. II that glutamic acid is present not only in the alkali hydrolysate of the iron-free compound but also in that of δ-N-hydroxy ornithine. It is apparent that the glutamic acid obtained on alkali hydrolysis of the iron-ffee compound is

the result of oxidation of the hydroxamate function, the proportion of glutamic acid to ornithine being determined by the amount ofthe hydroxamate function undergoing disproportionation under the hydrolytic conditions employed.

Quantitative analysis of the amino acid components.

A quantitative estimate of the amino acid components obtained under different hydrolytic conditions of the iron-free compound has been made with the use of the automatic amino acid analyser.

The amino acid composition of the acid hydrolysate of XFe and of the acid and alkali hydrolysates of the iron-free compound are presented in Table I. The results of acid hydrolysis of the iron-free compound obtained by resin treatment are also included in this table. The chromatographic patterns of the acid hydrolysate of XFe, acid hydrolysate of the iron-free compound and its reduction product, δ-N-hydroxy ornithine isolated from the acid hydrolysate of the iron-free compound from paper chromatograms and its reduction product, alkali hydrolysates of the iron-free compound and δ-N-hydroxy ornithine are depicted in fig V a-m.

It can be seen from Table I column A which gives the amino acid composition of the total hydrolysate of the iron-containing compound that detectable amounts of aspartic acid, glutamic acid, threonine, serine, glycine alanine, isoleucine and ornithine along with certain other unidentified ninhydrin positive compounds are present (fig. Vb & Vs). The total recovery of all the amino acids is approximately 1.5 moles per mole of XFe, of which 0.9 mole is accounted for by ornithine. All the other amino acids are present in equivalents of much less than 1 mole and therefore can only be regarded as products obtained by the disproportionation reaction undergone

by the hydroxamate function under acid conditions in presence of iron. The acid hydrolysate of the iron-free compound obtained by alkali treatment also contains aspartic acid, threonine, serine, glutamic acid, glycine, alanine leucine, isoleucine and ornithine (fig. Vc & VR). The yield of all these amino acids is considerably less as compared to that obtained in the case of the iron-containing compound (Table I - Column B). Other than these amino acids whose peak positions have been verified by comparison with those of a standard mixture of amino acids (fig. Va and Vf) an unknown compound is detected in peak position 29.3 ml in the basic run of the acid hydrolysate of the iron-free compound (fig. V h). Since δ-N-hydroxy ornithine has been known to be present in the hydrolysate, studies have been made to find out whether this new peak corresponds to δ-N-hydroxy ornithine. The hydrolysate has been subjected to catalytic reduction and the product is chromatographed as depicted in fig. Vi, *where* the analyses for the basic amino acids are presented. The analysis for the acidic and neutral are not included since no variations in the proportions of these amino acids have been detected on reduction. The peak in position 29.3 ml can definitely be attributed to δ-N-hydroxy ornithine for on reduction this peak completely disappears and the only amino acid that is formed is ornithine. This has been further confirmed by asimilar behaviour of δ-N-hydroxy ornithine isolated from an acid hydrolysate of the iron-free compound from paper chromatograms (fig. V j and Vk). Table I -column B gives the quantitative picture of the amino acids released on acid hydrolysis of the iron-free compound prepared by alkali treatment. The recovery of ornithine is 0.3 mole per mole of the compound (calculated for XFe). The peak corresponding to δ-N-hydroxy ornithine is generally not regular and the ninhydrin color value for this amino acid has not been calculated. Hence the concentration of δ-N-hydroxy ornithine has been calculated by quantitatively reducing the hydrolysate

By this method

the amount of δ-N-hydroxy ornithine present in the acid hydrolysate has been found to be 0.88 mole/mole of the compound (calculated for XFe).

The acid hydrolysis of the iron-free compound obtained by resin treatment (Table I -column c) yields only 0.17 mole of ornithine and 0.55 mole of δ-N-hydroxy ornithine. It has also been found that the proportions of ornithine and δ-N-hydroxy ornithine vary with slight changes in the hydrolytic conditions of the iron-free compound obtained by alkali or resin treatment. These variations can only be attributed to a probable interaction of the δ-N-hydroxy ornithine function with the unidentified components of this particular compound, since δ-N-hydroxy ornithine has been shown to be perfectly stable in other siderochromes under acid conditions in the absence of iron (7). In this context, it is significant to point out that the components so far identified to be present in the iron-binding compound isolated from N.crassa account for only 50% of the molecule.

The results of the amino acid analysis of the alkali hydrolysate of the iron-binding compound are presented in Table I column -D and fig V d and V i. In contrast to the results obtained on acid hydrolysis, the amino acid components of the alkali hydrolysate can be entirely accounted for by two amino acids namely ornithine (1.9 moles) and glutamic acid (0.9 moles). It has already been shown by paper chromatography (fig II) that δ-N-hydroxy ornithine (nitroindane dione salt) on alkali hydrolysis yields only ornithine and glutamic acid. A quantitative picture on the yields of ornithine and glutamic acid has not been possible due to the lack of weighable amounts of the authentic sample of δ-N-hydroxy ornithine. However, it can be seen from fig V m and Ve that the only amino acids obtained on alkali hydrolysis of an unknown amount of δ-N-hydroxy ornithine (nitro indane dione salt) are ornithine and glutamic acid. The yields of these two amino acids are quite

Table I.

Quantitative estimation of the amino acid components of the iron-binding compound released under different hydrolytic conditions.

The concentration of the amino acids are calculated from the chromatograms of the automatic amino acid analyser runs. All peaks large enough for quantitative estimation are indicated as mole/mole of xFe (M.wt 1228). The symbol "t" indicates that a very small peak is visible but is not large enough for any quantitative estimate. The symbol "0" indicates that there is no detectable deviation in the base line. The values given are corrected to the second decimal place.

Peak position (Effluent volume: ml)	Amino acid	Acid hydrolysis of xFe A	Acid hydrolysis of the iron free compound prepd by alkali treatment. B	Acid hydrolysis of the iron free compound prepd by resin treatment. C	ALKALI hydrolysis of the iron free compound prepd by alkali treatment. D
53.4	-	-	0.01	-	-
54.7	Aspartic acid	0.05	0.01	0.02	t
64.0	Threonine	t	t	0.01	0.01 (Broad Peak)
68.2	Serine	0.04	0.02	0.01	0
79.4	-	-	t	-	0.02
81.0	Glutamic acid	0.06	0.01	0	0.90
88.3	Proline	0	0	0	0
105.6	Glycine	0.12	0.09	0.08	0.14
113.9	Alanine	0	0.02	t	t
121.9	-	-	-	-	0.02
126.7	Half cysteine	0	0	0	0
147.2	Valine	0	0	0	0
196.1	-	0.04	-	-	-
199.0	Methionine	0	0	0	0
206.4	Isoleucine	0.02	t	0.02	0.02
210.6	Leucine	0.02	0.02	t	t
228.8	-	-	-	-	0.02
233.6	Tyrosine	0	0	0	0
239.4	Phenyl alanine	0	0	0	0
		Analysis for the basic amino acids			
29.3	- N- hydroxy-ornithine	0	0.88	0.55	Non-uniform yellow peak- not assayed
46.6	Ornithine	0.90	0.30	0.17	1.90
58.1	Histidine	0	0	0	0
72.5	Ammonia	-	-	-	-
119.5	Arginine	0	0	0	0

An unidentified broad peak in this region = 0.19

Fig. Va-m.

Quantitative amino acid analyses of the acid and alkali hydrolysates of the iron-binding compound isolated from N. crassa.

The amino acid analyses were carried out with the automatic amino acid analyser. Fig. V a-m have been drawn according to scale from the original runs. In the analysis of the basic amino acids present in a standard amino acid mixture (fig. Vf) the ninhydrin was let in 20 min. after the start of the run. The recorder was put on 40 min. after the start of the run. In all the other runs the ninhydrin pump and the recorder were started at zero time. The iron-free compound used in these experiments was prepared from xFe by alkali treatment.

Fig. Va.

Chromatogram of a standard mixture of acidic and neutral amino acids.

The standard mixture contained 0.5 μ mole of each amino acid.

Absorbance
0·8
1·0
0·8
0·6
0·4
0·3
0·2
0·1
Effluent volume: ml.
54·7
64·0
68·2
81·0
88·3
105·6
113·9
126·7
147·2
199·0
206·4
210·6
233·6
239·4
ASPARTIC ACID
THREONINE
SERINE
GLUTAMIC ACID
PROLINE
GLYCINE
ALANINE
CYSTINE
VALINE
METHIONINE
ISOLEUCINE
LEUCINE
TYROSINE
PHENYL ALANINE

FIG. Va

Fig. Vb.

Chromatogram of the acidic and neutral amino acids present in the acid hydrolysate of xFe.

The acid hydrolysate from 3.7 μ mole of xFe was placed on the column.

Fig. Vc.

Chromatogram of the acidic and neutral amino acids present in the acid hydrolysate of the iron-free compound.

The acid hydrolysate of the iron-free compound prepared from 4.3 μ mole of xFe was placed on the column.

Absorbance
0·1
0·2
0·3
0·4
0·5
0·6
0·8
1·0
8
ASPARTIC ACID
SERINE
GLUTAMIC ACID
GLYCINE
ISOLEUCINE
LEUCINE
547
640
682
810
883
1056
1139
1267
1472
1961
1990
2064
2106
2336
2394
Effluent volume. ml.

FIG. V b

Absorbance
0·1
0·2
0·3
0·4
0·5
0·6
0·8
1·0
8
ASPARTIC ACID
SERINE
GLUTAMIC ACID
GLYCINE
ALANINE
ISOLEUCINE
LEUCINE
547
640
682
810
883
1056
1139
1267
1472
1990
2064
2106
2336
2394
Effluent volume. ml.

FIG. V c

Fig. Vd.

Chromatogram of the acidic and neutral amino acids present in the alkali hydrolysate of the iron-free compound.

The alkali hydrolysate of the iron-free compound prepared from 4.3 μ mole of xFe was placed on the column.

Fig. Ve.

Chromatogram of the acidic and neutral amino acids present in the alkali hydrolysate of δ - N - hydroxy ornithine (nitro-indane dione salt).

The alkali hydrolysate from an unknown amount of δ - N - hydroxy ornithine was placed on the column.

Absorbance
∞
1·0
0·8
0·6
0·5
0·4
0·3
0·2
0·1
GLUTAMIC ACID
GLYCINE
ISOLEUCINE
LEUCINE
54·7
64·0
68·2
81·0
88·3
105·6
113·9
126·7
147·2
199·0
206·4
210·6
233·6
239·4
Effluent volume ml

FIG. V d

Absorbance
∞
1·0
0·8
0·6
0·5
0·4
0·3
0·2
0·1
GLUTAMIC ACID
54·7
64·0
68·2
81·0
88·3
105·6
113·9
126·7
147·2
199·0
206·4
210·6
233·6
239·4
Effluent volume ml.

FIG. V e

Fig. Vf.

Chromatogram of a standard mixture of basic amino acids

The standard mixture contained 0.5 μ mole of each amino acid.

Fig. Vg.

Chromatogram of the basic amino acids present in the acid hydrolysate of xFe.

The acid hydrolysate from 0.8 μ mole of xFe was placed on the column.

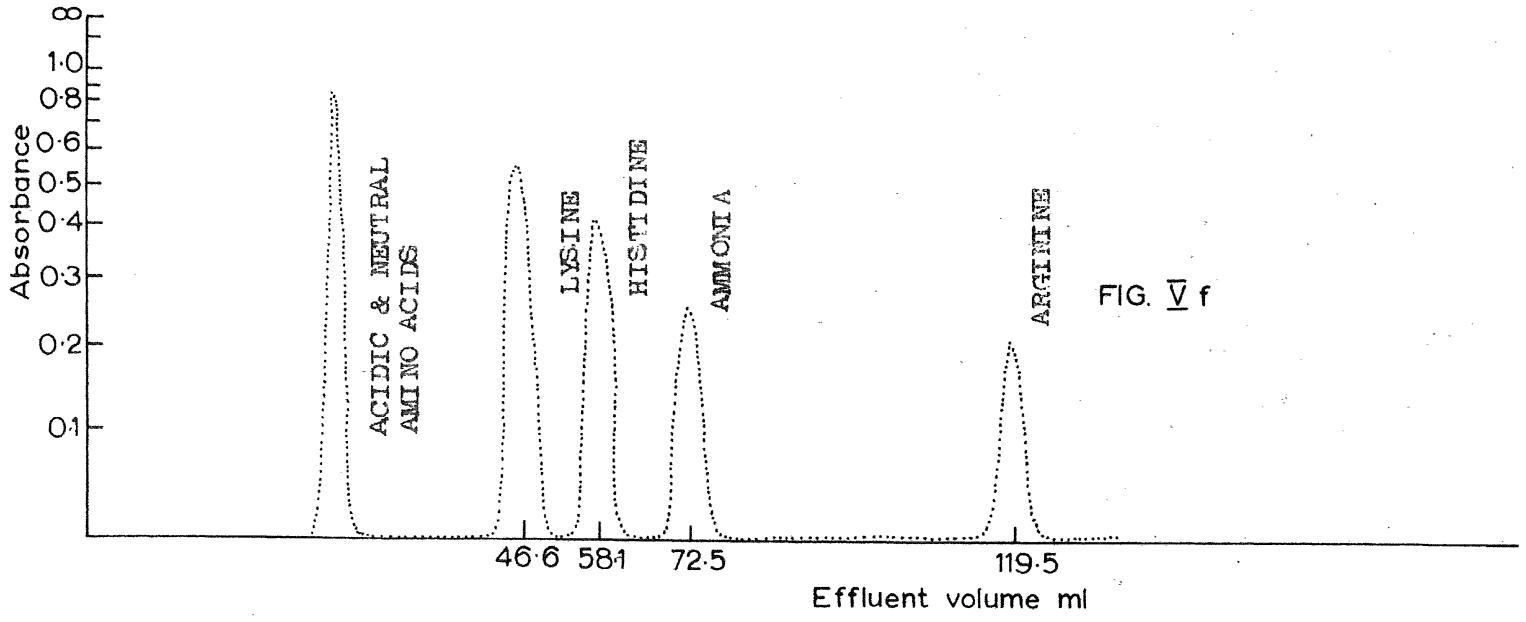

Absorbance
1·0
0·8
0·6
0·5
0·4
0·3
0·2
0·1
ACIDIC & NEUTRAL AMINO ACIDS
LYSINE
HISTIDINE
AMMONIA
ARGININE
46·6
58·1
72·5
119·5
Effluent volume ml

FIG. V f

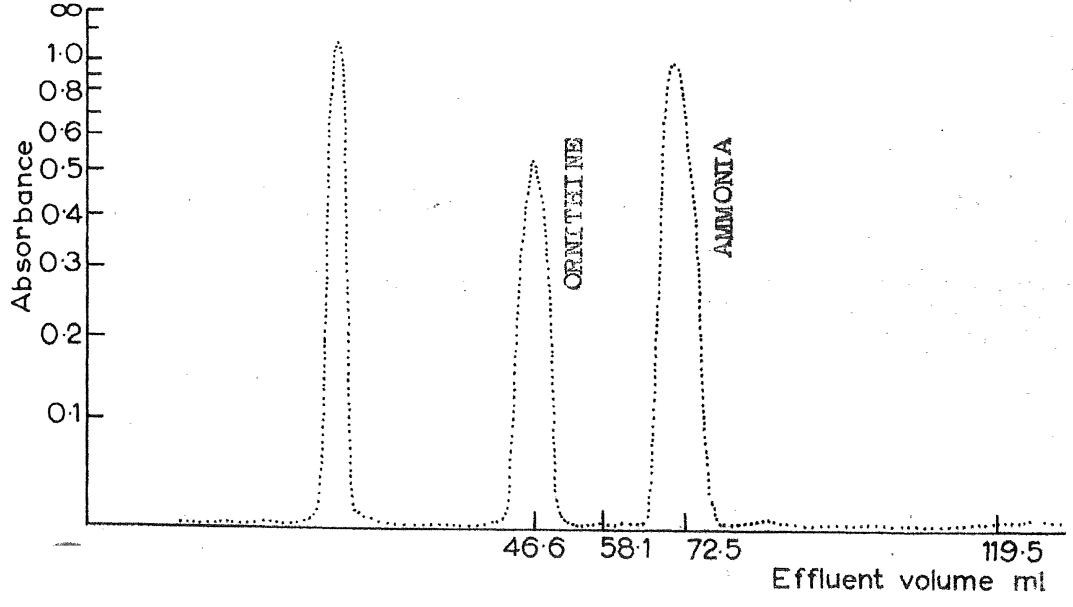

Absorbance
1·0
0·8
0·6
0·5
0·4
0·3
0·2
0·1
ORNITHINE
AMMONIA
46·6
58·1
72·5
119·5
Effluent volume ml

FIG. V g

Fig. V.

Chromatogram of the basic amino acids present in the acid hydrolysate of the iron-free compound.

The acid hydrolysate of the iron-free compound prepared from 1.2 μ mole of xFe was placed on the column.

Fig. Vi.

Chromatogram of the basic amino acids present in the reduced acid hydrolysate of the iron-free compound.

The reduced acid hydrolysate of the iron-free compound prepared from 1.2 μ mole of xFe was placed on the column.

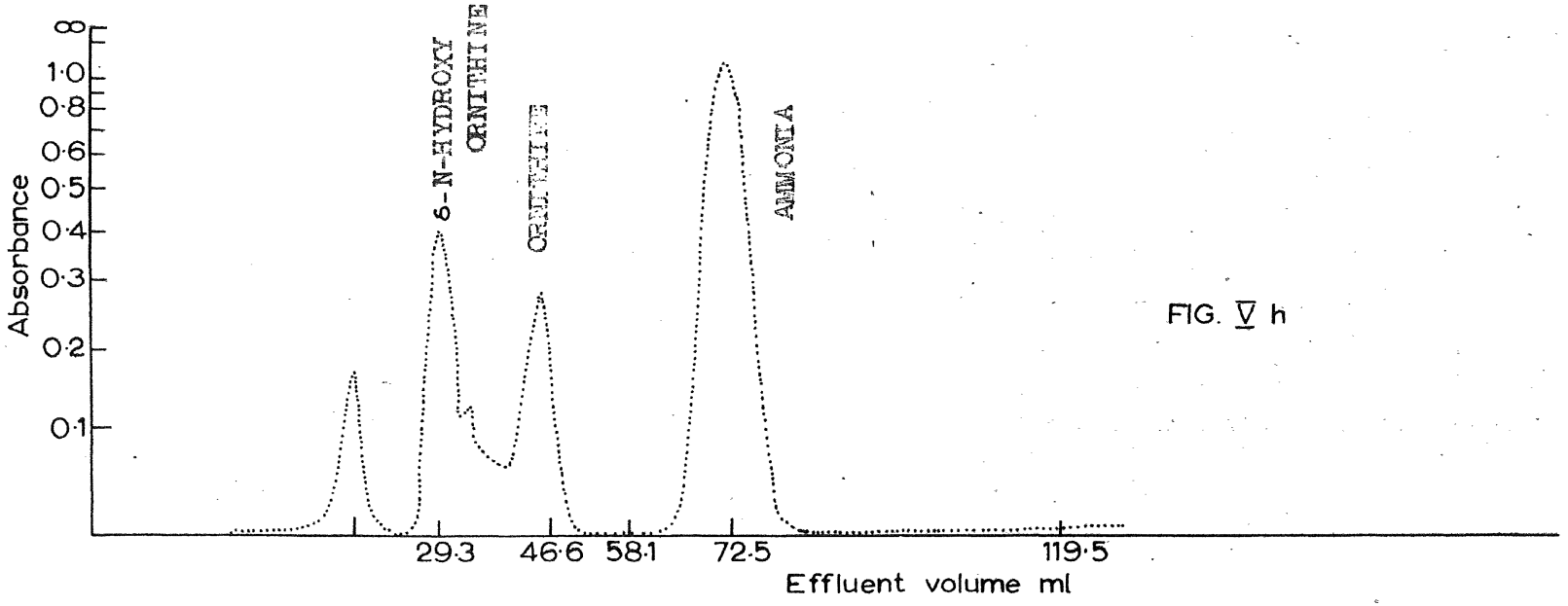

Absorbance
1·0
0·8
0·6
0·5
0·4
0·3
0·2
0·1
δ-N-HYDROXY ORNITHINE
ORNITHINE
AMMONIA
29·3
46·6
58·1
72·5
119·5
Effluent volume ml

FIG. V h

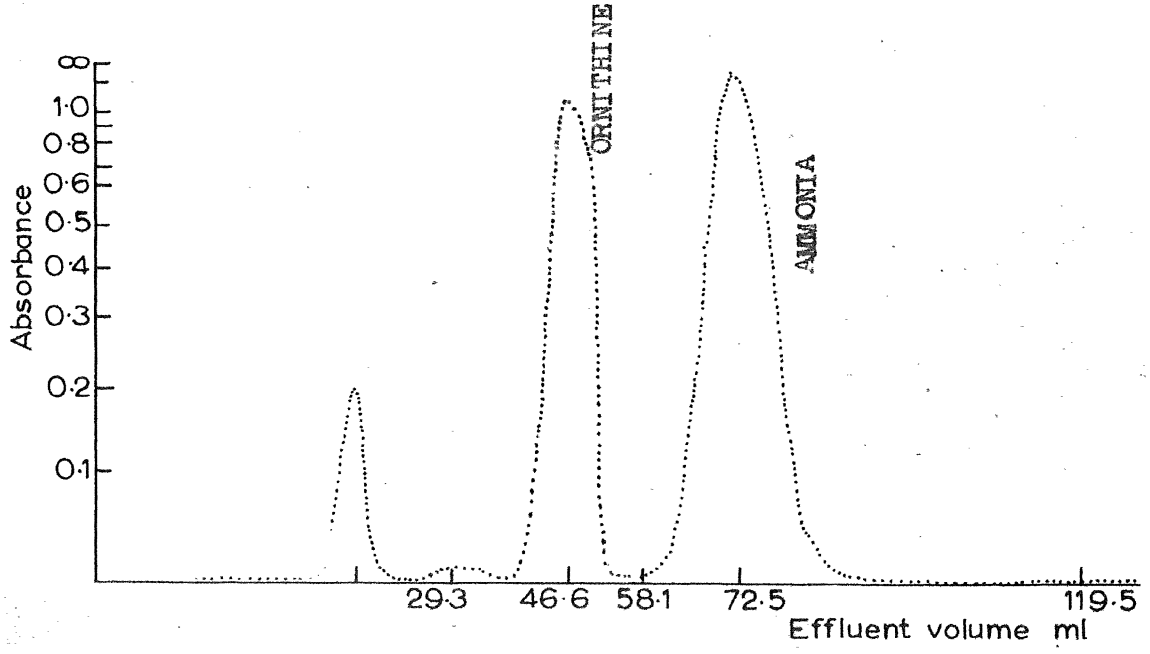

Absorbance
1·0
0·8
0·6
0·5
0·4
0·3
0·2
0·1
ORNITHINE
AMMONIA
29·3
46·6
58·1
72·5
119·5
Effluent volume ml

FIG. V i

Fig. Vj.

Chromatogram of δ - N - hydroxy ornithine isolated by paper chromatography from the acid hydrolysate of the iron-free compound.

An unknown amount of the preparation was placed on the column.

Fig. Vk.

Chromatogram of the reduction product of the isolated δ - N - hydroxy ornithine.

An unknown but the same amount of the preparation used in the experiment depicted in Fig. Vj. was quantitatively reduced and placed on the column.

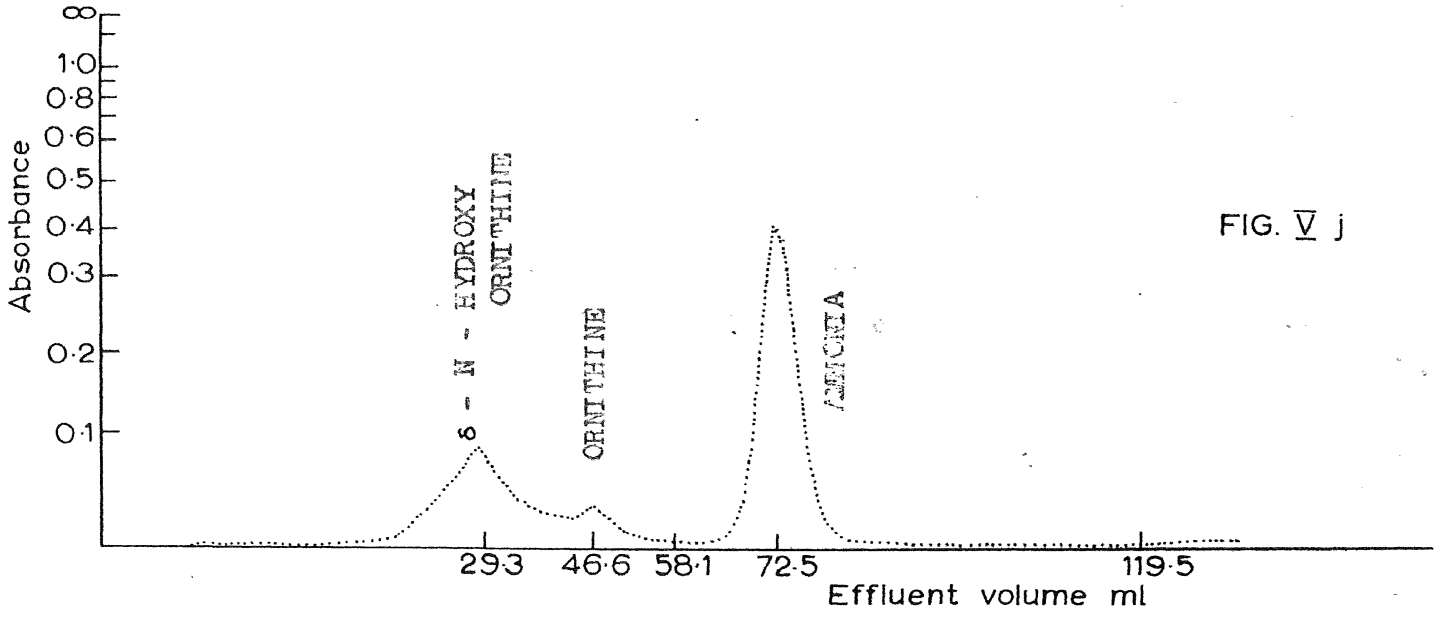

Absorbance
1·0
0·8
0·6
0·5
0·4
0·3
0·2
0·1
δ - N - HYDROXY ORNITHINE
ORNITHINE
AMMONIA
29·3
46·6
58·1
72·5
119·5
Effluent volume ml

FIG. V j

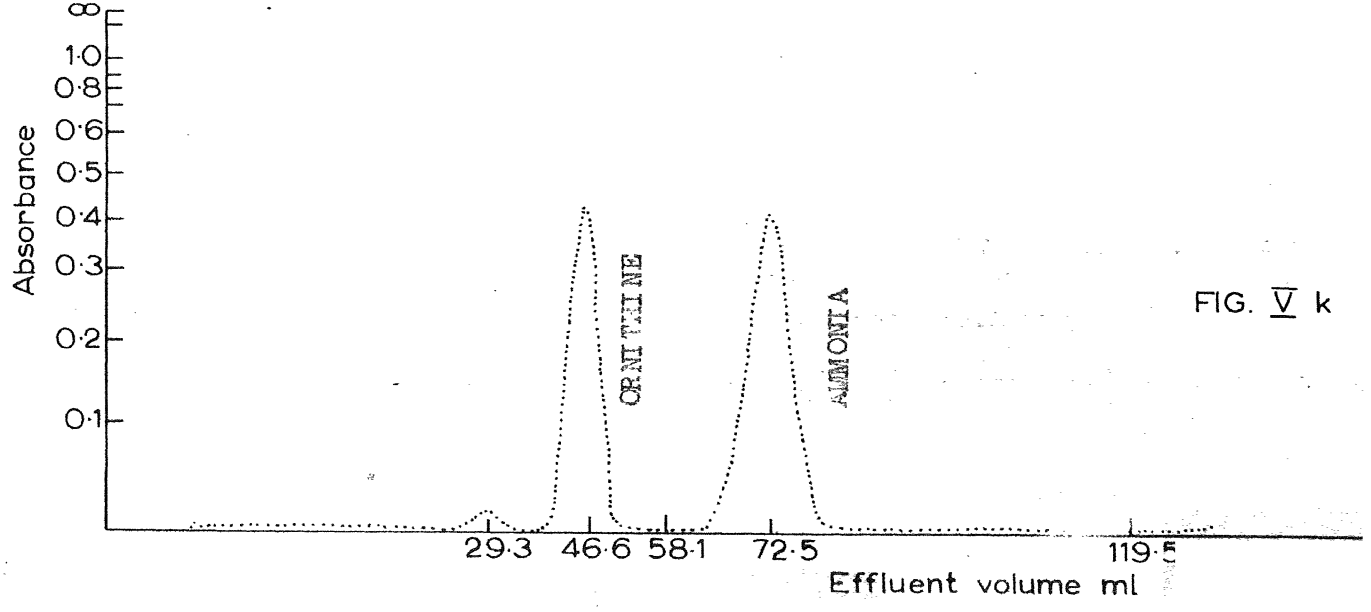

Absorbance
1·0
0·8
0·6
0·5
0·4
0·3
0·2
0·1
ORNITHINE
AMMONIA
29·3
46·6
58·1
72·5
119·5
Effluent volume ml

FIG. V k

Fig. Vl.

Chromatogram of the basic amino acids present in the alkali hydrolysate of the iron-free compound.

The alkali hydrolysate of the iron-free compound prepared from 1.1 μ mole of xFe was placed on the column.

Fig. Vm.

Chromatogram of the basic amino acids present in the alkali hydrolysate of δ - N - hydroxy ornithine (nitro indane dione salt).

The alkali hydrolysate from an unknown but the same amount of δ - N-hydroxy ornithine (nitro indane dione salt) used in the experiment depicted in fig. Ve was placed on the column.

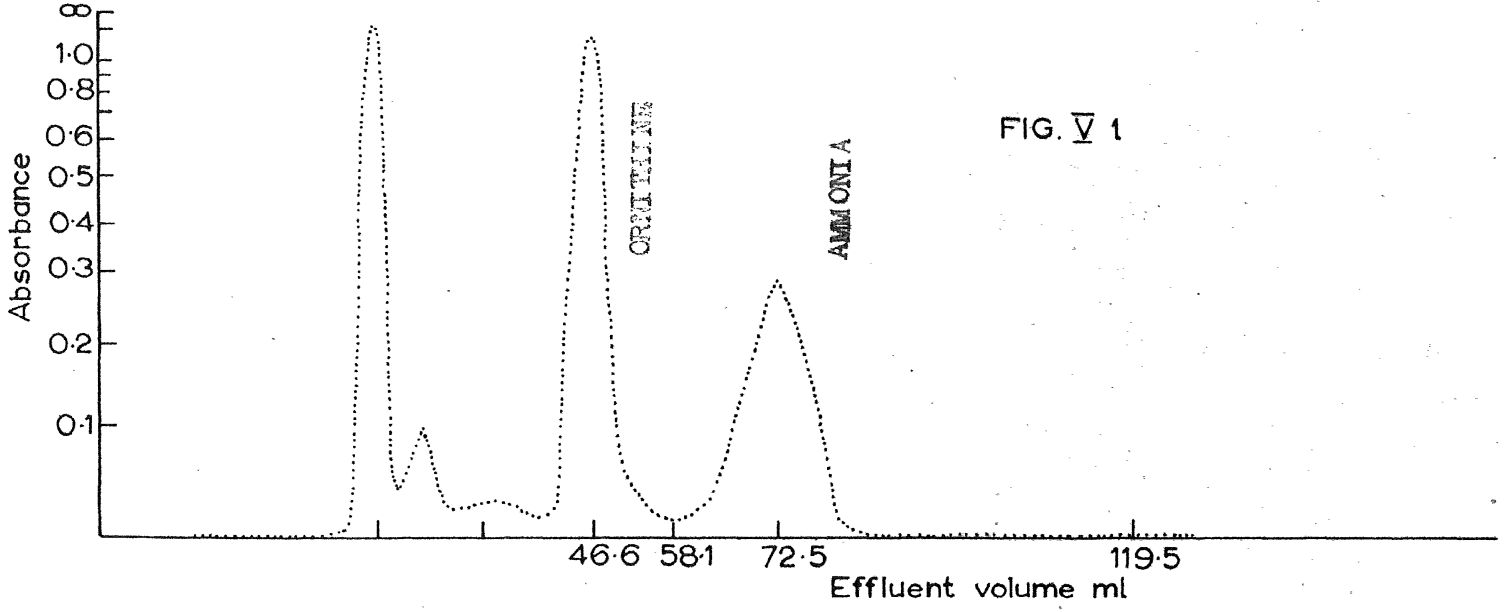

Absorbance
1·0
0·8
0·6
0·5
0·4
0·3
0·2
0·1
ORNITHINE
AMMONIA
46·6
58·1
72·5
119·5
Effluent volume ml

FIG. V 1

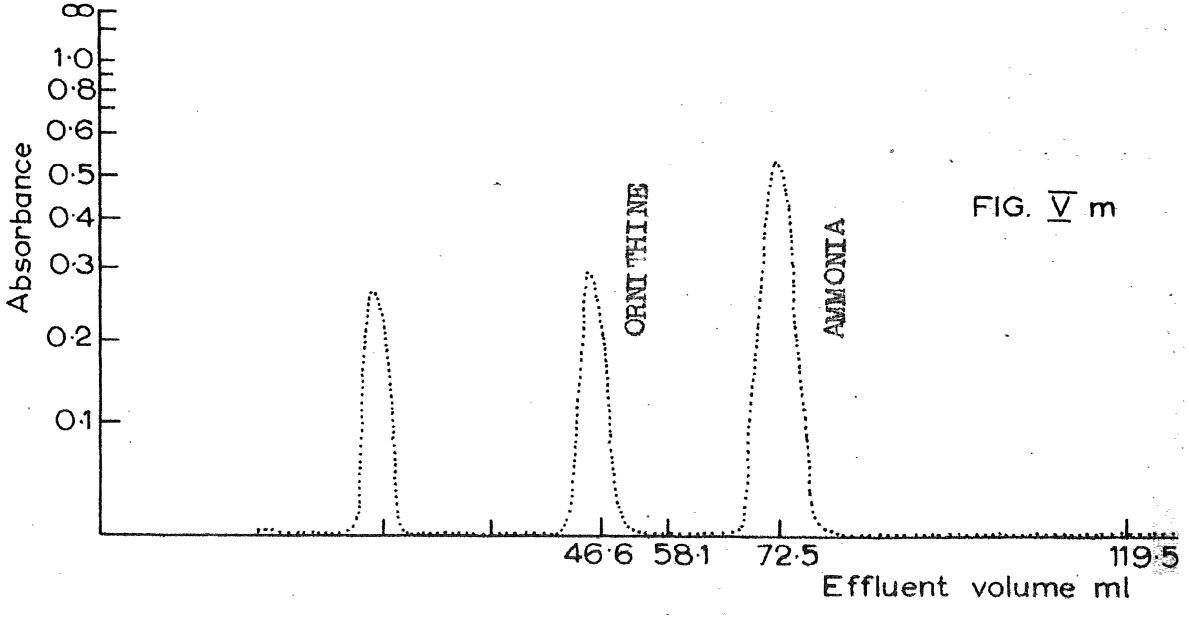

Absorbance
1·0
0·8
0·6
0·5
0·4
0·3
0·2
0·1
ORNITHINE
AMMONIA
46·6
58·1
72·5
119·5
Effluent volume ml

FIG. V m

significant but their ratio cannot be assumed to be the same for the δ-N-hydroxy ornithine in the chemically bound form in which it is present in the N. crassa siderochrome. The facts that variable and small yields of ornithine have been obtained with only traces of glutamic acid in the acid hydrolysate of the iron-free compound indicate that these two amino acids are most probably not present as such in this molecule but are derived from δ-N-hydroxy ornithine. It appears that quantitative yields of these two amino acids are obtained only under alkaline conditions. If such is the case, it can be seen that glutamic acid and ornithine are present to the extent of 3 moles per mole of XFe and account for the total amount of δ-N-hydroxy ornithine present.

DISCUSSION

In Table II a comparison has been made between the degradation products of ferrichrome and ferrichrome A with those of the N. crassa compound. The structure proposed for the ferrichromes is depicted in fig VI. Acid hydrolysis of the iron-free ferrichrome yields 3 moles of δ-N-hydroxy ornithine and 3 moles of glycine. Under these conditions ferrichrome A gives 3 moles of δ-N-hydroxy ornithine, 2 moles of serine and 1 mole of glycine (7). However, the N. crassa siderochrome yields variable and smaller amounts of δ-N-hydroxy ornithine and ornithine. This type of a result cannot be attributed to the presence of iron, since the alkali as well as the resin treatments have been found to remove the iron completely from XFe. Ferrichrome which is also a neutral compound like XFe, gives quantitative yields of δ-N-hydroxy ornithine when the iron is removed by alkali treatment.

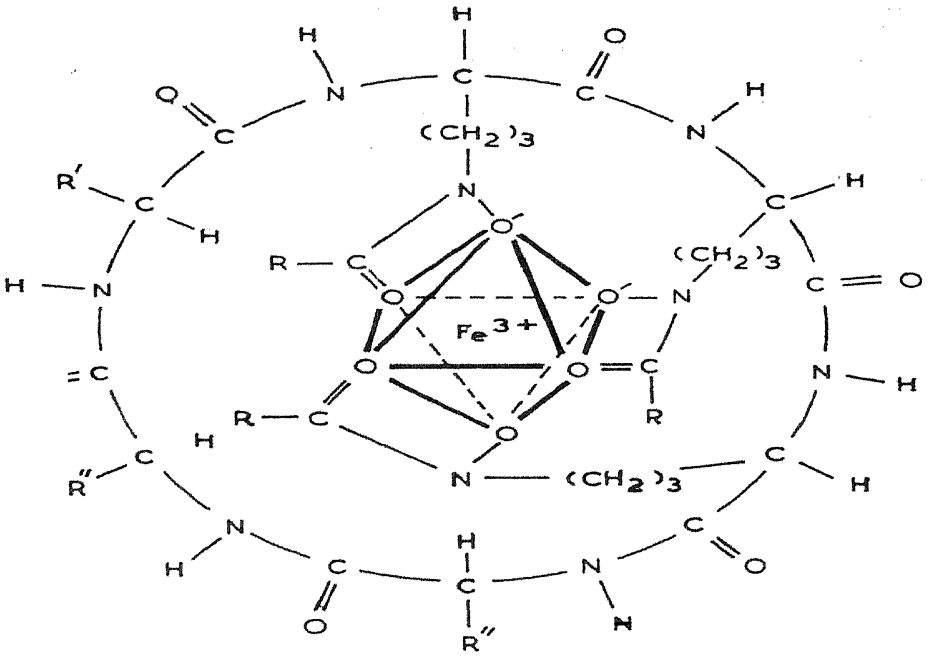
Periodate treatment of the iron-free moieties of ferrichrome and ferrichrome A yields 3 moles of acetic acid and 3 moles of β-methyl glutaconic

Table II. A comparison between the degradation products of ferrichrome, ferrichrome A and XFe

Compound	Acid hydrolysis	Periodate treatment	Alkali hydrolysis
Iron-free ferrichrome	δ-N-hydroxy ornithine (3 moles) + glycine (3 moles)	Acetic acid (3 moles)	Ornithine, glycine and large amounts of acetic acid.
Iron-free ferrichrome A	δ-N-hydroxy ornithine (3 moles) + glycine ((1 mole) and serine (2 moles)	β-methyl glutaconic acid (3 moles)	-
Iron-free *N. crassa* compound	δ-N-hydroxy ornithine and ornithine (variable amounts)	β-methyl glutaconic acid or a closely related compound (1 mole)	Ornithine (2 moles and glutamic acid (1 mole)

E.VI

Structural model for the Ferrichromes (9)



Ferrichrome : $R = CH_3^-$; $R' = R'' = H$

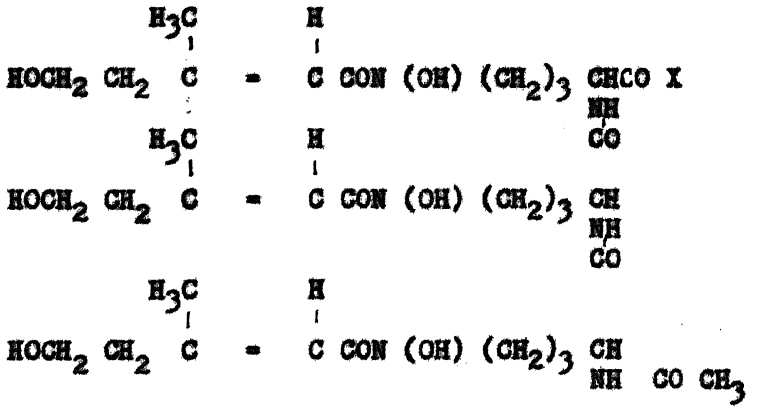
Ferrichrome A : $R = HOOC-CH_2-C(=CH-C-)-CH_3$; $R'=H$; $R''=HOCH_2^-$

acid respectively (3). In the case of XFe, only one mole of β-methyl glutaconic acid or a compound closely related to it has been detected and the presence of volatile acids in this compound cannot be ruled out at present.

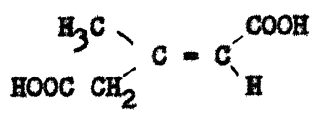
The yield of only one mole of β-methyl glutaconic acid or a closely compound and the smaller and variable yields of δ-N-hydroxy ornithine on acid hydrolysis can be the result of an interference by the unidentified portion of this compound which constitutes nearly 50% of this molecule. This unidentified portion which probably contributes to the slight yellow color of the iron-free compound can very well be the main characterestic component distinguishing it from the other known siderochromes. So far no knowledge has been gained as to the nature of this yet unidentified component.

Alkali hydrolysis of ferrichrome has been reported to yield large amounts of acetic acid in addition to glycine and ornithine.(8). However, a detailed study on the alkali hydrolysis of ferrichrome has not been reported. It has been found in the present study that the N. crassa siderochrome as well as an authentic sample of δ-N-hydroxy ornithine (nitroindane dione salt) yield significant amounts of glutamic acid and ornithine on alkali hydrolysis. It is of interest to report here that glutamic acid has been reported to be a product of performic acid oxidation of δ-N-hydroxy ornithine(9). It has also been indicated that a non-enzymic disproportionation of the sensitive hydroxylamino group would yield both glutamic acid and ornithine(10). It is concluded that the combined yields of ornithine and glutamic acid obtained on alkali hydrolysis of the iron-free compound of N.crassa accounts for the total amount of δ-N-hydroxy ornithine present in the molecule, namely 3 moles.

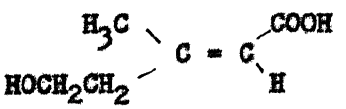
It is also of interest here to consider the amino acid and other constituents of certain other siderochromes, sideramines in particular, presented in Table III. It can be easily seen that the N. crassa compound is different from the ferrioxamines or the ferrichrome class of compounds considering the amounts and nature of the degradation products obtained. Recently it has been reported that coprogen has been isolated from N. crassa (SHEAR et B.O. DODGE) grown under conditions of iron deficiency and a tentative structure has also been proposed for this compound (11) as given below.



Coprogen has been found to contain 3 moles of δ-N-hydroxy ornithine, 3 moles of trans-5-hydroxy- 3 methyl-pentene - 2 enoic acid which is very closely related to β-methyl glutaconic acid as can be seen from the formulae depicted below.



Trans-β-methyl glutaconic acid



Trans-5 hydroxy- 3 methyl-pentene- 2 enoic acid

In addition coprogen has also been indicated to contain one mole

Table III. A comparison between the degradation products of ferrioxamines and ferrichrome class of compounds.

Siderochrome	Hydroxamate function	Other amino acids	Organic acid.
Ferrioxamines			
A_1	1-amino-5-hydroxylamino pentane -I (2 moles)		Acetic acid (1 mole)
	1-amino-4-hydroxylamino butane -II	-	Succinic acid (2 moles)
A_2	I (1 mole) II (2 moles)	-	Acetic acid (1 mole) Succinic acid (2 moles)
B	I (3 moles) II (nil)	-	Acetic acid (1 mole) Succinic acid (2 moles)
D_1	I (3 moles) II (nil)		Acetic acid (2moles) Succinic acid (2 moles)
D_2	I (2 moles) II (½ mole)		Acetic acid (nil) Succinic acid (3 moles)
E	I (3 moles) II (nil)	-	Acetic acid (nil) Succinic acid (3 moles)
G	I (3 moles) II (nil)	-	Acetic acid (nil) Succinic acid (3 moles)
Ferrichrome class			
Ferrichrysin	δ-N-hydroxy ornithine-III (3 moles)	-	Acetic acid (3 moles
Ferrihodin	III (3 moles)	serine (2 moles) glycine (1 mole)	Cis-5-hydroxy-3 - methyl pentene-2-enoic acid(3 moles)
Ferrirubin	III (3 moles)	serine (2 moles) glycine (1 mole)	Trans-5-hydroxy-3 methyl pentene-2-enoic acid(3 moles)

of acetic acid and an unidentified component X. The studies so far carried out on the iron-binding compound isolated from N. crassa Em 5297a (wild) under conditions of cobalt toxicity reveal that it may be related to coprogen but shows certain distinguishing features. They are :

1. Quantitative yields of δ-N-hydroxy ornithine are not obtained on acid hydrolysis of the iron-free compound employed in the present study.

2. Only one mole of β-methyl glutaconic acid or a closely related compound has been obtained on periodate treatment.

3. Coprogen has been reported to have an absorption band at 252 -254 mμ (12) in the ultra violet which has not been detected in the XFe absorption spectrum (fig I - Section A).

4. The iron content of XFe has been found to be 4.48%. The iron content of coprogen has been reported to be 6.64%.

Finally it may be mentioned that the iron-binding compound used in the present study has not been obtained in a crystalline form although its homogeneity has been established by paper chromatography and paper electrophoresis in several solvent systems. The quantitative yields of glutamic acid and ornithine obtained on alkali hydrolysis, the combined amounts of which can account for 3 moles of δ-N-hydroxy ornithine which is necessary for the formation of a highly stable polyhydroxamate iron-complex as has been found to be the case in the ferrichromes and other siderochromes indicates that the molecular weight assigned for XFe is in the correct range. Under these circumstances, the smaller yields of δ-N-hydroxy ornithine obtained on acid hydrolysis of the iron-free compound on a molar basis, appears to be due to the interference of a highly reactive unidentified component present as an integral part of the molecule.

SUMMARY

1. The iron-binding compound (X) isolated as the iron-complex (XFe) has been found to contain 4.48% iron and on this basis the molecular weight of the compound is 1228.

2. The iron can be removed from XFe effectively by alkali or resin treatments.

3. Periodate treatment of the iron-free compound yields 1 mole of β-methyl glutaconic acid or a compound closely related to it.

4. Acid hydrolysis of XFe yields variable proportions of several amino acids, due to the disproportionation reaction of the hydroxamate function, present in this compound, in presence of iron under acid conditions.

5. Acid hydrolysis of the iron-free compound yields variable yields of ornithine and δ-N-hydroxy ornithine.

6. Alkali hydrolysis of the iron-free compound yields 2 moles of ornithine and 1 mole of glutamic acid. Alkali hydrolysis of an authentic sample of δ-N-hydroxy ornithine yields only ornithine and glutamic acid.

7. It has been concluded that the combined yields of the products of alkali hydrolysis of the iron-free compound represent the amount of δ-N-hydroxy ornithine present in the molecule, namely 3 moles.

8. The smaller yields of δ-N-hydroxy ornithine obtained on acid hydrolysis of the iron-free compound has been attributed to a probable interfering effect of the unidentified integral part of this compound which constitutes nearly 50% of the molecule.

9. A comparison has been made between the degradation products obtained from XFe with those of the known siderochromes (mainly sideramines) and

the N. crassa compound appears different though closely related to the others.

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CHAPTER II

METABOLIC STUDIES ON THE IRON-BINDING COMPOUND ISOLATED FROM

METABOLIC STUDIES ON THE IRON-BINDING COMPOUND ISOLATED FROM NEUROSPORA CRASSA

The isolation of a large number of iron-binding or iron-containing compounds like ferrichrome, ferrichrome A, ferrioxamines, terregen̲s factor including the sideromycins (1,2) from microorganisms has led to investigations relating to the transport mechanisms involved in iron metabolism. Further, detailed studies with ferrichrome, isolated from the smut fungus Ustilago sphaerogena (3) have established that this organic iron can act as an iron donor for heme synthesis. It has been demonstrated that ferrichrome and related compounds can act as growth factors for Arthrobacter JG 9, Pilobolus kleinii, and for certain other organisms, whose requirements can be satisfied by a comparatively high amount of hemin (1). Subsequently, it has been shown in the case of Arthrobacter JG 9 that it has an obligatory requirement for ferrichrome in order to maintain normal growth and catalase activity, even though inorganic iron may be provided in the medium (4). Barnham (5) has observed that ferrichrome -Fe^{59} can get incorporated into catalase and that this process can be repressed by the addition of hemin in growing cultures of Arthrobacter JG 9. He has further demonstrated that cell-free extracts of Rhodopseudomonas spheroides are able to synthesise hemin when incubated with an oxidizable substrate, protoporphyrin IX and iron provided as ferrichrome (6). The striking evidence that these compounds exert a role in iron metabolism has been the capacity of several microorganisms to secrete specific iron-binding compounds into the culture fluid when grown under conditions of iron deficiency (1,7). The iron-complexes of several of these compounds have basic similarities with the ferrichromes such as possessing a hydroxamate structure at the iron-binding site and exhibiting

a mutual replaceability of one with another as a growth factor for certain microorganisms, despite a difference in overall structure.

Earlier (Chapter I) it has been reported that a new iron-binding compound has been isolated from iron-deficient or cobalt-toxic cultures of Neurospora crassa and that the new compound can be differentiated from ferrichrome, ferrichrome A and others on the basis of chromatographic mobilities, solubility properties and amino acid composition. But evidences such as its strong binding affinity for ferric but not for ferrous iron, the presence of δ-N-hydroxy ornithine and the detection of β-methyl glutaconic acid indicate its similarity to the sideramines. Metabolic studies with ferrichrome have been mostly confined to an organism like Athrobacter JG 9 which has no capacity to synthesise ferrichrome type compounds, but responds strikingly to extraneously added ferrichrome. But, primarily these compounds should prove metabolically useful to parent organisms producing them, in their iron metabolism. The results obtained in this context with N. crassa are presented in this chapter.

EXPERIMENTAL

Materials

Fe^{59}-citrate and $Fe^{59}Cl_3$ were obtained from Atomic Energy Commission, Trombay, India. Analytical grade salts were used for constituting the N. crassa basal medium. For the assay of aconitase, cis-aconitic anhydride was prepared from trans-aconitic acid.

Organism

A wild strain of Neurospora crassa Em 5297a as mentioned in Chapter I was used in these studies.

Medium and growth conditions.

The composition of the basal medium and the procedure employed for the preparation of iron deficient media have been described in Chapter I. The organism was generally grown in 10 ml basal medium in 50 ml pyrex conical flasks in stationary cultures at pH 4.8.

Isolation and assay of the iron-binding compound

The iron-binding compound (X) was isolated as the iron-complex (XFe or XFe^{59}) from iron-deficient culture fluid of N. crassa as described in Chapter I. The final preparation obtained was chromatographically and electrophoretically pure and contained all the iron in bound form. The relative production of the iron-binding compound under different conditions was assessed by the procedure of Neilands (1) by adding 1 ml of iron solution (1 mg/ml) to 3 ml culture fluid and measuring the optical density of the supernatant obtained after centrifugation, at 440 mμ.

Fe^{59}-citrate (or $Fe^{59}Cl_3$) and XFe^{59} uptake studies by iron-deficient mycelia

The iron-binding compound was isolated as Fe^{59} labelled complex (XFe^{59}) and used for uptake studies. Fe^{59} citrate (or $Fe^{59}Cl_3$) and XFe^{59} were added to 40 hr old iron-deficient cultures of N. crassa at 10 μg Fe/10 ml medium level in 0.1 ml asceptically. To some flasks containing Fe^{59}-citrate (or $Fe^{59}Cl_3$) the iron-free fraction prepared from XFe by alkali treatment (Chapter I) was added after subsequent neutralization. The flasks were then transferred to a reciprocal shaker and at periodic intervals the mycelia and the culture fluid were removed to assess radioactivity and disappearance of XFe^{59} from the media respectively. The radioactivity left over in the medium was also assessed. The mycelia were washed free of

adhering radioactivity, dried in an oven at 60° overnight and then digested with acid. The acid digests were used to measure radioactivity. The disappearance of XFe^{59} from the medium was assessed by measuring the optical density of the culture fluid at 440 mμ.

Radioactivity measurements were made in a DSS-5 scintillation detector attached to a decade scaler (type 151A, Nuclear Chicago Corporation, Des Plaines, Illinois, U.S.A.). Appropriate corrections due to background and radioactive decay were applied. The error due to count rate was less than ± 2%. Fe^{59} was counted at the operation voltage of 1150 V.

Enzyme assays

Preparation of enzyme extracts.

The mycelia grown for the required length of time were washed with ice-cold water to free the same from adhering media, gently pressed between folds of filter paper and then ground to a fine suspension in a mortar with glass powder and phosphate buffer (pH 7.0; 0.05 M). The extract was centrifuged at 3000 x g for 15 min. and the precipitate was washed once with the buffer and the supernatants were pooled. All the operations were carried out at 4°. Aliquots of this preparation were used for the enzyme assays.

Enzyme assays

Catalase.

The method described by Ramachandran and Sarma (8) was used. 1.0 ml of the enzyme extract was incubated with 1.0 ml of 0.2 N H_2O_2 at 30° in a 50 ml pyrex conical flask. At the end of 5 min. the reaction was stopped with 5.0 ml of 5 N. H_2SO_4 and the left over H_2O_2 was determined by titration against 0.01 M. $KMnO_4$. The enzyme activities of the extracts were always

preadjusted by dilution such that the amount of substrate decomposed did not exceed 20% of the total amount added to the reaction mixture. The enzyme activity is expressed as ml 0.01 M $KMnO_4$ consumed/mg protein/5 min.

Succinic dehydrogenase

The method of Green et al (9) was followed which involved the decolorisation of dichlorophenol indophenol in presence of cyanide to inhibit cytochrome oxidase. The incubation mixture which contained in 3 ml, 0.75 ml of 0.2 M (pH 6.8) phosphate buffer, 0.15 ml of 0.05% 2:6 dichlorophenol indophenol, 0.5 ml enzyme, 0.1 ml of 0.05 M KCN, 0.2 ml of a solution containing 1 mg egg albumin, 1.2 ml of water and 0.1 ml of 0.4 M sodium succinate was incubated at 30°. The fall in the optical density of the dye was followed and the activity is expressed as fall in optical density at 600 mμ/3 min/mg protein.

Aconitase

The method described by Morrison (10) was followed which involved the estimation of citric acid formed by the enzymic hydrolysis of cis-aconitic anhydride. To 4.0 ml of water, 0.5 ml of a solution containing 20 μ moles of cis-aconitic anhydride was added followed by 0.5 ml of the enzyme solution after 5 min. After 15 min. the reaction was stopped with 1.0 ml of 50% TCA, centrifuged and suitable aliquots from the supernatant were taken to estimate citric acid. The enzyme activity is expressed as μg citric acid formed/ mg protein/15 min.

Estimation of citric acid

The method described by Stern (11) was followed. To 10 -100 μg of citric acid in 1.0 ml volume in a test tube 5 drops of M. KBr followed by 10 drops of 5% $KMnO_4$ were added with shaking to ensure mixing. The solution was allowed to stand for 10 min during which time excess of permanganate,

indicated by the purple color, was maintained. The test tube was placed in ice water and the excess permanganate was decolorised by the dropwise addition of 6% H_2O_2, care being taken to see that excess of H_2O_2 was not added. The pentabromoacetone formed was extracted with 2.5 ml of heptane. 2.0 ml of the heptane layer was transferred to a test tube and 4.0 ml of thiourea solution (2 gm of sodium borate dissolved in 100 ml of 4% thiourea; pH 9.2) was added. After shaking for 10 min. the aqueous layer was drawn and the color was read at 430 mμ. Appropriate blanks and standards were run.

Protein estimation in enzyme extracts

The procedure employed was that of Lowry et al (12) using Folin-Ciocalteu reagent (13). A 1% solution of copper sulphate was mixed with an equal volume of a 2% solution of sodium potassium tartrate (reagent A). A 2% solution of sodium carbonate in 0.1 N NaOH was mixed with reagent A in the ratio of 50:1 by volume (reagent B). 0.1 ml of enzyme extract, diluted suitably to contain protein in the range 10 - 200 μg was treated with 5.0 ml of reagent B and after 10 min. 0.5 ml of Folin reagent was added and shaken immediately. 30 min after the addition of the Folin reagent, the blue color developed was measured against a suitable blank using a Klett-Summerson Photoelectric Colorimeter with filter No. 66. A standard solution of crystalline bovine serum albumin (Armour and Co., U.S.A.) was used as the protein standard.

Preparation of Folin-Ciocalteu reagent (13)

In a 2 litre round bottomed flask, the following were mixed in the order and in amounts indicated: Sodium tungstate (100 g); sodium molybdate (25 g), distilled water (700 ml), syrupy phosphoric acid (50 ml), con. HCl (100 ml)

The contents were refluxed for 10 hr and on cooling, 150 g of lithium sulphate, 50 ml of distilled water and 4-5 drops of bromine water were added, boiled for 15 min, to expell the bromine and diluted suitably to give an acidity of 1.0 N.

RESULTS

The iron-binding compound has been detected in the culture fluid of N. crassa only under conditions of iron deficiency whether direct or conditioned due to cobalt toxicity (Chapter I). To find out the responde of the production of this compound to the iron concentration of the medium, the organism has been grown at different levels of iron. The production of the iron-binding compound, catalase activity and growth have been determined under these conditions and from the results presented in Table I, it is clear that whereas the catalase activity progressively decreases with decreasing iron concentration in the medium, the production of the iron-binding compound shows a corresponding increase. Growth itself is not strikingly affected under these conditions. The fact that even at zero addition of iron to the basic iron deficient medium there is nearly 50% of normal growth, indicates that the growth medium though should be very low in iron content can support appreciable growth even though catalase activity and the production of the iron-binding compound are profoundly influenced.

In view of the fact that catalase activity and the iron binding compound production are strikingly controlled by the iron status of the medium in N. crassa, it has been of interest to examine the primary site affected in iron deficiency in this organism. It is clear from the results presented in Table II that at 24 hr, mycelial growth and catalase activity are normal under iron deficient conditions, although subsequently the specific

Table I. Effect of iron status of the medium on the production of the iron binding compound, catalase activity and growth of *N. crassa*

levels of iron have been added to a basic iron deficient media. The experimental details are given in text.

Iron added (µg)	Iron-binding compound produced O.D. at 440 mµ	Catalase activity ml 0.01 M $KMnO_4$ consumed/mg protein/ 5	Growth mg dry wt.
-	0.24	8.0	22.8
0.05	0.26	10.1	24.6
0.10	0.22	12.7	28.6
0.20	0.20	19.3	32.6
0.50	0.11	31.2	39.0
1.00	0.03	54.0	45.0

Table II. Effect of iron deficiency on the production of the iron-binding compound catalase activity and growth, as a function of growth period in N. crassa.

The experimental details are given in text.

Period	Normal media			Iron deficient media		
hr	Iron-binding compound O.D at 440 mμ	Catalase activity ml 0.01M $KMnO_4$ consumed/mg protein/ 5 min.	Growth mg dry wt	Iron-binding compound O.D. at 440 mμ	Catalase activity ml 0.01M $KMnO_4$ consumed/mg protein/5 min	Growth mg dry wt
24	-	6.1	6.0	0.04	7.0	5.9
48	-	42.7	30.5	0.10	8.5	19.5
72	0.03	55.0	42.5	0.24	10.2	24.0

activity of catalase falls very much below the control values under these conditions. It is significant that the iron-binding compound is secreted into the medium even at 24 hr growth under iron deficient conditions, when overall growth and catalase activity are not affected.

It has been reported (Chapter I)that the new iron-binding compound from N. crassa binds iron with great affinity. It is of interest to study the role of XFe as an iron source to N. crassa. Iron supplements in the form of XFe, ferrichrome, ferrichrome A, ferric acet hydroxamate and ferric chloride have been added to the basic iron deficient medium and the potency of each to maintain normal growth and catalase activity has been examined. Each supplement has been provided at a level of 1 µg Fe/10 ml of the iron deficient medium. The results are presented in Table III. It is evident that XFe is as potent as inorganic iron and ferrichrome in maintaing normal growth and catalase activity and the organism can utilize the iron of XFe even though it is held very firmly in the chemical

If XFe acts as a general iron donor to the organism, it influence the activities of not only heme enzymes, but also of the non-heme iron enzymes which may be affected in iron deficiency. To make the medium strictly iron-free, the organism has been grown in the basic iron deficient medium for 40 hr, by which time iron deficiency has set in as indicated by a fall in not only catalase activity but also in the activities of non-heme iron enzymes like succinic dehydrogenase and aconitase. Iron as $FeCl_3$ and XFe has been added at 40 hr growth to the iron deficient cultures and all the three enzyme activities as well as growth have been estimated at 72 hr growth. The results presented in Table IV indicate that the addition of inorganic iron as well as XFe restores the depressed enzyme levels to the same extent.

Table III. Effect of XFe, Ferrichrome, Ferrichrome A, Ferric acethydroxamate and inorganic iron on growth and catalase activity of N. crassa.

The compounds have been added at a level of 1 µg iron/10 ml iron deficient medium. The experimental details are given in text.

Compound added	Growth mg dry wt	Catalase activity ml 0.01M $KMnO_4$ consumed/ mg protein/5 min.
Nil	24.5	13.4
XFe	40.4	53.9
Ferrichrome	42.5	55.6
Ferrichrome A	32.8	33.8
Ferric acethydroxamate	35.0	37.0
$FeCl_3 . 6H_2O$	44.4	56.1

Table IV. Effect of inorganic iron and XFe added to iron-deficient cultures at 40 hr. growth, on Catalase, Succinic dehydrogenase, Aconitase and growth of M. crassa

The additons have been made at a level of 1 µg Fe/10 ml medium. The results of enzymic activities are expressed as percentages of the values recorded for 40 hr normal mycelia which are taken as 100. The actual values are given in parantheses.

The experimental details are given in text.

Treatment	Catalase activity ml 0.01M $KMnO_4$ consumed/mg protein/ 5 min.	Succinic dehydrogenase activity-fall in O.D. at 600mµ/ mg protein/3 min.	Aconitase activity µg citric acid mg protein/ 15 min	Growth mg dry wt.
40 hr Normal	100 (38.5)	100 (0.10)	100 (97.0)	25.2
40 hr iron-deficient	25	70	84	18.0
72 hr Normal	138	120	70	46.0
72 hr iron-deficient	30	40	40	26.0
XFe added at 40 hr	110	92	62	42.0
Iron added at 40 hr	119	87	60	44.0

Having established the metabolic potency of XFe as an iron donor, the interest has been to see whether XFe is permeable to the cell or the iron is splitt off extracellularly and then incorporated. XFe^{59} has been provided at 10 µg Fe/10 ml medium to 40 hr old iron-deficient cultures and the flasks are shaken in a reciprocal shaker. At different intervals of time, the incorporation of radioactivity into the mycelium and the disappearance of XFe^{59} from the medium, by measuring the fall in O.D. at 440 mµ, have been assessed. Similar uptake studies have also been conducted when iron is provided as Fe^{59} citrate (or $Fe^{59}Cl_3$). The results presented in Table V indicate that whereas XFe^{59} incorporation reaches a maximum within 15 min. that of Fe^{59} citrate takes nearly 2 hr to reach a similar level of incorporation. When iron is removed from XFe by alkali treatment (Chapter I) and the iron-free fraction is added to Fe^{59} citrate after neutralization, again a similar enhanced rate of Fe^{59} incorporation is observed as compared to that when Fe^{59} citrate alone is used. The mycelial weights remain more or less constant throughout the incubation period. $Fe^{59}Cl_3$ incorporation follows the same pattern as that of Fe^{59}citrate. To further substantiate that XFe has been taken in as an intact molecule, the mycelia just after 5 min. incubation with XFe^{59}, by which time the Fe^{59} taken in would not have been dissipated to other systems appreciably, have been washed well, extracted with phosphate buffer and the buffer extract processed as for XFe isolation (Chapter I). The final preparation chromatographed on paper in two different solvent systems gives a radioactive spot corresponding to XFe and accounting for 60% of the total iron uptake. Studies with identical mycelia provided with Fe^{59}citrate and processed under identical conditions indicate that the spot corresponding to XFe can account for only 10% of the total radioactivity incorporated. This 10% may be due to the formation of the complex in the mycelia as well as due to the small amount

Table V.

XFe^{59} and Fe^{59}citrate uptake by 40 hr. old iron-deficient mycelia of N. crassa

The additions have been made at 10 µg Fe/10 ml medium in 0.1 ml volume to 40 hr old iron-deficient cultures. The experimental details are given in text.

Incubation time (min)	XFe^{59} uptake: Radioactivity in mycelium cpm/mycelium (x 10^4)	XFe^{59} uptake: Radioactivity in medium cpm/10 ml medium (x 10^4)	O.D. of medium at 440mµ	Fe^{59}citrate uptake: Radioactivity in mycelium cpm/mycelium (x 10^4)	Fe^{59}citrate uptake: Radioactivity in medium cpm/10 ml medium (x 10^4)	Fe^{59}citrate uptake in presence of iron-free compound: Radioactivity in mycelium cpm/mycelium (x 10^4)	Fe^{59}citrate uptake in presence of iron-free compound: Radioactivity in medium cpm/10 ml medium (x 10^4)
0	-	2.29	0.130	-	2.29	-	2.29
15	1.76	0.44	0.010	0.40	1.87	1.75	0.44
30	1.85	0.35	0.008	0.72	1.53	1.81	0.40
45	1.83	0.37	0.008	0.99	1.28	1.81	0.42
60	1.89	0.32	0.008	1.25	0.95	1.85	0.30
120	1.91	0.29	0.008	1.86	0.35	1.86	0.36

From the radioactivity actually detected in the mycelium and that left over in medium, the losses due to washing of mycelium and other manipulations have been assessed. The loss is about 5 - 7% of the actual radioactivity expected on the mycelium (calculated from the fall of radioactivity in the medium) and is in the same range in all cases.

Table VI. Detection of XFe^{59} in the mycelium

40 hr. old iron-deficient mycelia after incubation with XFe^{59} and citrate added at 10 µg Fe/10 ml medium, have been processed after thorough washing to remove adhering radioactivity and the final preparation chromatographed on paper in two solvent systems. The radioactivity put in has been adjusted to give 2.29×10^4 counts/min/10 ml medium.

The experimental details are given in text.

Compound added	Radioactivity in mycelium counts/min/ mycelium	Radioactivity in XFe spot	
		Butanol:Acetic acid: water. Rf = 0.56 counts/min	Methanol:water Rf = 0.88 counts/min
XFe^{59}	6080	3600	3480
Fe^{59} citrate	1560	150	175

of the iron-binding compound already present in the 40 hr old iron-deficient culture fluid. These results are presented in Table VI.

Within the short time (15 min) of maximal XFe^{59} incorporation and disappearance from the medium, no possible break down products can be detected and the radioactivity incorporated cannot be exchanged when reincubated in a fresh medium containing cold XFE or inorganic iron.

DISCUSSION

The production of the iron-binding compound in N. crassa is strikingly dependent on the iron concentration of the medium as is the case with the production of the other iron-binding compounds reported by Neilands(1). Progressive increase in the production of the iron-binding compound and a corresponding decrease in catalase levels with the fall in the iron status of the medium establishes the dependency of both the systems on iron nutrition and a reciprocal relationship between the two.

Ferrichrome is the natural iron-containing metabolite of Ustilago sphaerogena and ferrichrome - A is the iron-binding moiety secreted under conditions of iron deficiency by the same organism (3,15). Burnham and Neilands (4) on screening several microorganisms for ferrichrome activity have found that N. crassa (16117) gives a strongly positive reaction and answers as well the test for bound hydroxylamine. Preliminary studies with N. crassa Em 5297a indicate that two iron chelates could be isolated from mycelia grown under normal conditions, one having an Rf value corresponding to ferrichrome and the other to XFE. Both the chelates on acid hydrolysis answer for hydroxyl amine. These preliminary data are presented in Table VII and it can be seen that the iron-chelates put together account nearly for

of the total iron taken in by the organism.

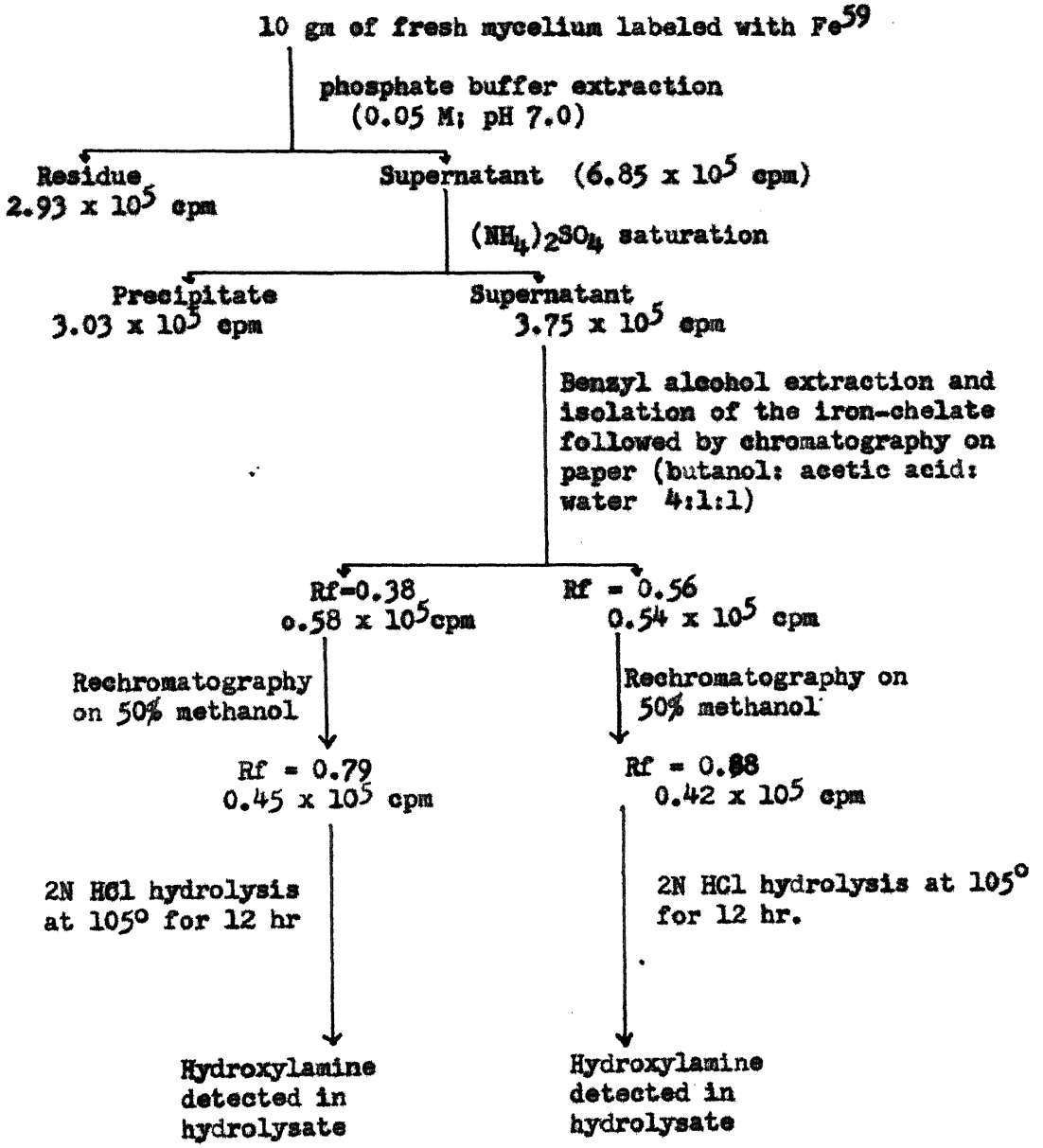
The secretion of the iron-binding moiety under deficient conditions can be explained as due to the unavialbility of iron for the formation of the natural chelates. If like ferrichrome, the N. crassa iron chelate acts as the natural iron donor for heme synthesis in this organism, the secretion of the iron-binding moiety would precede the fall in catalase activity when the organism is grown under iron deficient conditions as is illustrated in data presented in Table II.

The survival value of the phenomenon of iron-binding compound secretion to the parent organism is established by the metabolic potency of XFe to act as an iron donor to maintain normal growth and catalase activity, even though the iron is held strongly in the chemical sense (Table III). This is further emphasised by a certain specificity shown by the organism as regards the nature of the organic iron it can utilize. Whereas ferrichrome and XFe serve as good sources of iron, ferrichrome A and ferric acethydroxamate do not serve equally well, when all these are provided at the same iron level (Table IV). The nutritional inactivity of ferrichrome A has already been noted with Arthrobacter JG 9 (4) and it should be of exclusive importance to the parent organism

work implicating ferrichrome in iron sequestration and transport has been mainly confined to the role of these compounds in supplying iron for heme synthesis. Burnham (5) has envisaged a possibility that in JG 9 there can be two routes for iron incorporation, one to the heme through the ferrichrome and the other representing the non-heme iron. But studies with XFe in the present investigation have revealed that when it is provided as the sole iron source, it not only can support normal growth and catalase activity but also influence the levels of non-heme iron enzymes like succinic dehydrogenase and aconitase. While not much is known regarding

Table VII - Isolation of iron-chelates from normal mycelia of N. crassa

N. crassa mycelia grown for 72 hr with Fe^{59}-citrate in normal media were pooled, extracted with phosphate buffer and buffer extract after saturation with $(NH_4)_2SO_4$ processed as for XFe isolation as described in Chapter I.



the active center of these enzymes and the role of iron in influencing the activities of these enzymes may be indirect, it is clear that XFe can supply iron for such a role. At least in the parent organism N. crassa, the organic iron chelate eleborated by it can assume a generalized role controlling iron supply to heme as well as non-heme iron enzyme systems. Even in Arthrobacter JG 9 where it has been established that ferrichrome influences heme synthesis, the effects of ferrichrome deprivation on non-heme iron enzyme systems may give rise to interesting results.

The last evidence cited to indicate the metabolic potency of XFe is the striking avidity with which the iron deficient mycelium takes up XFe as compared to the rates of entry of inorganic iron or a simple chelate like ferric citrate. It is not clear whether this is due to a preferential incorporation of the biologically active chelate (Tables V and VI). A parallel situation has also been met with in tomato plants where ferrioxamin B translocation to the upper parts of the plants takes place more rapidly than ionic iron (16).

Finally, the isolation of the natural iron chelates elaborated by N. crassa when grown under normal conditions with optimal levels of iron and their participation in iron metabolism of the parent organism await further investigation. Such a study is warranted by the metabolic potency of XFe, isolated under iron deficient conditions.

SUMMARY

1. Neurospora crassa Em 5297a secretes increasing concentrations of the iron-binding compound (X) with decrease in the iron concentration of growth medium from optimal levels. Catalase activity shows a decrease under these conditions.

2. Under iron deficient conditions the production of the iron-bindin compound precedes the fall in catalase activity.

3. The iron-complex of the iron-binding compound (XFe) can act as a good iron source for the organism to maintain normal growth and catalase activity. While ferrichrome is equally effective, ferrichrome A and ferric acet hydroxamate are only partially beneficial.

4. XFe also influences non-heme iron enzymes like succinic dehydrogenase and aconitase, when provided as the sole iron source.

5. XFe is permeable to N. crassa mycelia and is incorporated at a much faster rate as compared to that of inorganic iron or a simple chelate like ferric citrate.

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CHAPTER III

COBALT TOXICITY AND IRON METABOLISM IN *NEUROSPORA CRASSA*

COBALT TOXICITY AND IRON METABOLISM

Cobalt toxicity has been related to a conditioned iron deficiency in (1). A direct cobalt-iron antagonism is demonstrable in crassa as well (2). Healy, Cheng and McElroy (3) as a result of studies at the enzymic level have concluded that cobalt toxicity is iron deficiency in N. crassa. A further striking evidence for a cobalt-iron antagonism in this organism has been the isolation of an iron-binding compound (X) from the culture fluid when the organism is grown under conditions of straight iron deficiency or cobalt toxicity (Chapter I). The iron-complex of the iron-binding compound (XFe) has been found to serve as a good source of iron for the parent organism to maintain normal growth, catalase and a few non-heme iron enzyme activities. Further, this organic iron gets incorporated into the mycelium at a much faster rate as compared to inorganic iron or a simple chelate like ferric citrate (Chapter II). Studies have also indicated that XFe belongs to the siderochrome (4) class of compounds (Chapter I).

In view of these observations, a detailed study on the interference of cobalt with the iron metabolism of the mold N. crassa has been carried out and the results are presented in this Chapter.

Materials.

Carrier-free Fe^{59} citrate and $Fe^{59}Cl_3$ were Energy , Trombay, India. Glycine-2-C^{14} was obtained from the , Amersham, U.K. Protoporphyrin IX and recrystalli hemin were purchased from Mann Research Company. XFe and

isolated both from cobalt-toxic and iron-deficient culture fluids of N. crassa.

Culture and growth conditions

N. crassa Em 5297a (wild) was used in these studies. The organism was grown in 50 ml pyrex conical flasks in 10 ml medium at 30° in stationary cultures. The composition of the medium has been described in Chapter I.

Effect of cobalt on Fe^{59} uptake.

The organism was grown in presence of 800 µg cobalt/10 ml basal medium and optimal levels of iron (1 µg Fe/10 ml basal medium) as Fe^{59}-citrate. After the organism had grown for the required period, the mycelia were removed, washed free of adhering radioactivity and the fresh weights were recorded. Each mycelium was made into a fine suspension with 5 ml of water. An aliquot was used to measure the total radioactivity incorporated. The rest of the aliquot was precipitated with cold trichloroacetic acid (10% in final concentration). The precipitate was washed 4 times with cold trichloroacetic acid and the radioactivity on the precipitate was measured after digestion with acid. The trichloroacetic acid precipitable Fe^{59} has been referred to as bound Fe^{59}.

Effect of cobalt on Fe^{59} and glycine -2-C^{14} incorporation into the mycelial heme fraction.

The mycelia grown in presence of cobalt and the appropriate tracer were washed free of adhering radioactivity and the hemin was isolated from the mycelial acetone powder according to the following method of Labbe and Nishida (5).

The mycelial acetone powder was prepared by homogenising the

mycelia with 5 vol. of chilled acetone in a waring blendor. The resulting slurry was filtered through a Buchner funnel, washed with an excess of chilled acetone, and the residue spread out on filter paper and allowed to dry. The acetone powder was stored in a desiccator at 4°. 1 g of the acetone powder was suspended in 5 ml of water and 100 ml of a solvent mixture of acetic acid saturated with strontium chloride and acetone (1:3) was added. Since sufficient hemin could not be isolated to permit subsequent purification 50 mg of carrier hemin was added. The mixture was allowed to stand overnight and then briefly heated to the boiling point of the solvent and filtered. The residue was washed thrice with 10 ml aliquots of the acetic acid:acetone mixture and the filtrate was heated slowly to 102°. Hemin started crystallizing on concentration and was allowed to be completed over a period of 24 hr. The hemin crystals were collected by centrifugation and washed successively with 50% acetic acid and water, each washing being repeated twice. The crystals were further washed once with alcohol and finally with ether. Recrystallization of hemin was carried out by dissolving the final preparation in pyridine-chloroform (1:3) and filtering into acetic acid saturated with strontium chloride. The filter paper was washed with small amounts of chloroform till free of color. Hemin was isolated and washed as before. The hemin sample was then dissolved in alkaline pyridine and aliquots were taken for measuring radioactivity and hemin content. The radioactivity on a known amount of hemin from a known weight of the mycelium has been taken as a measure of the Fe^{59} or glycine-2-C^{14} incorporation into the mycelial heme fraction.

Effect of cobalt on Fe^{59} incorporation into protoporphyrin in cell-free extracts

The procedure employed was essentially the same as described by

Nishida and Labbe (6). 72 hr. old normal mycelia were ground in 0.1 M phosphate buffer (pH 7.5) in presence of tween 20 and glass powder using a pestle and mortar and centrifuged at 15,000 x g for 20 min. after keeping the homogenate stirred for an hour. This enzyme preparation was incubated with the other constituents in amounts as used by Labbe and Hubbard (7) for 2 hr. under nitrogen at 37° in a Dubnoff metabolic shaker. The composition of the incubation mixture was : Iron ($Fe^{59}SO_4$) - 24 mμ moles; cobalt ($Co^{60}Cl_2$)- 30 mμ moles; Protoporphyrin - 30 mμ moles; Tris (pH 7.8) - 180 μmoles; Ascorbic acid - 40 μmoles; Enzyme - 0.5 ml containing 6 mg protein/ml. The iron-binding fraction prepared from XFe by alkali treatment (Chapter I) was added after neutralisation, in 0.1 ml and in slight excess equivalents of the iron present in the incubation mixture. The reaction was stopped with acetic acid: acetone mixture and hemin was isolated and recrystallised from the incubation mixture after adding 10 ml carrier blood by the procedure indicated earlier. The recrystallized hemin was dissolved in alkaline pyridine and aliquots were used to measure radioactivity and hemin content. When iron incorporation from intact XFe^{59} was studied under these conditions, it was found necessary to use higher levels of substrates owing to the low specific activity of the isolated XFe^{59}. The incubation mixture in 5 ml contained: Iron ($Fe^{59}SO_4$ or XFe^{59}) - 0.5 μ mole; Protoporphyrin - 0.6 μmole; Tris (pH 7.8) - 360 μ moles; Ascorbic acid - 80 μ mole; Enzyme - 2 ml containing 6 mg protein/ml.

Radioactive measurements

C^{14} measurements were made in a Geiger-Muller end window counter attached to a decade scaler (type 151A, Nuclear-Chicago Corporation, Desplaines, Illinois, U.S.A.). Appropriate corrections due to backgrowund and self absorption were applied. Fe^{59} measurements were carried out in

a well-type scintillation detector attached to a decade scaler. due to radioactive decay were taken into account in Fe^{59} measurement. The error due to count rate was within ± 2%.

Catalase assay

Catalase activity was determined in the mycelial phosphate buffer extracts by the procedure described in Chapter II. Protein content was measured according to Lowry, Rosebrough, Farr and Randall (8).

Estimation of the iron-binding compound

The production of the iron-binding compound was estimated as described in Chapter II by adding 1 ml of $FeCl_3 . 6 H_2O$ solution (1 mg Fe/ml) to 3 ml of culture filtrate. The supernatant obtained after centrifugation was measured at 440 mμ.

RESULTS

It has been indicated that a reciprocal relationship exists in N. crassa between the production of the iron-binding compound and catalase activity and this is governed by the iron status of the growth medium (Chapter II). It can be seen from data presented in Table I that increasing concentrations of cobalt cause an increase in the production of the iron-binding compound and a corresponding fall in the catalase activity. The fall in the production of the iron-binding compound at the highest level of cobalt employed can be explained as due to the severe inhibition of the growth of the organism.

Thus, the results presented above simulate conditions of straight iron deficiency and it has been of interest to examine whether cobalt causes an iron deficiency by inhibiting the uptake of the essential metal

Table I. Effect of increasing concentrations of cobalt on growth, catalase activity and iron-binding compound production in N. grassa at the end of 72 hr growth.

The experimental details are given in text.

Cobalt added µg/10 ml medium	Growth mg dry wt	Catalase activity ml 0.01 M $KMnO_4$ consumed/mg protein/ 5 min	Iron-binding compound O.D. at 440 mµ
-	42.8	50.2	0.03
50	46.0	35.4	0.10
200	37.2	20.2	0.16
600	28.1	13.1	0.20
800	22.0	9.0	0.22
1000	13.2	5.7	0.07

Table II Effect of cobalt on total and bound- Fe^{59} in the mycelia as a function of growth period in *N. crassa*

Fe^{59} was included to give 14.00×10^3 counts/min/10 ml basal medium.

The experimental details are given in text

Period of growth hr	Normal			Cobalt toxic		
	Total Fe^{59} counts/min/ mycelium $\times 10^2$	Total Fe^{59} counts/min/ 100 mg dry wt. $\times 10^2$	Bound Fe^{59} counts/min/ 100 mg dry wt. $\times 10^2$	Total Fe^{59} counts/min/ mycelium $\times 10^2$	Total Fe^{59} counts/min/ 100 mg dry wt. $\times 10^2$	Bound Fe^{59} counts/min/ 100 mg dry wt. $\times 10^2$
24	59	502	83	106	4364	355
30	70	334	82	105	1710	119
48	108	301	77	100	669	56
54	115	253	73	101	583	49
72	113	239	72	103	420	45

by the organism. The results presented in Table II indicate that cobalt does not inhibit iron uptake but enhances instead the rate of incorporation, when the organism is grown in presence of toxic levels of cobalt and optimal levels of iron. The Fe^{59} incorporated per cobalt-toxic mycelium is double the corresponding value for the normal mycelium at the end of 24 hr of growth. This indicates that cobalt per se enhances the rate of Fe^{59} incorporation into the mycelium in addition to its growth inhibitory effects, which is also a contributory factor to the increased iron concentration, when the results are expressed on a unit mycelial weight basis. However, bound Fe^{59} shows a significant decrease at the end of the growth period (72 hr) in cobalt toxic mycelia as compared to the normal mycelia indicating that the cobalt-iron antagonism is intracellular in nature in this organism. In order to detect the primary site of induced iron deficiency, the effects of cobalt on growth, catalase activity, iron-binding compound production, bound Fe^{59} and heme Fe^{59} in the mycelia have been determined as a function of the growth period. From the results presented in Tables II and III, it is evident that at 24 hr of growth, in presence of cobalt, catalase activity, bound Fe^{59} and heme Fe^{59} values are comparable to that of the normal mycelia. The iron-binding compound can be detected in the culture fluid even at this period of growth under cobalt toxic conditions. The subsequent decrease in bound Fe^{59} and heme Fe^{59} thus indicates an effect of cobalt at the level of iron utilization for the synthesis of key metabolites.

The fall in heme Fe^{59} values can be due to two factors namely an effect of cobalt on the synthesis of the porphyrin moiety and a possible competition between cobalt and iron at the level of incorporation into the porphyrin nucleus. The former aspect has been examined by growing

the organism under cobalt toxic conditions in presence of
The results presented in Table IV indicate that at the end of 72 hr growth cobalt does not inhibit total glycine -2-C^{14} incorporation into the mycelia but inhibits incorporation into the heme fraction.

The effect of cobalt on iron incorporation into protoporphyrin has been studied in cell-free extracts. Earlier (Chapter II) it has been found that XFe can act as a good source of iron for N. crassa and is incorporated at a much faster rate into the mycelium as compared to inorganic iron or a simple chelate like ferric citrate. Further, the incorporation of the latter is markedly increased when the iron-binding fraction prepared from XFe is added. Hence, it has been of interest to study the effect of the iron-binding fraction on iron incorporation into protoporphyrin and the effect of cobalt on it, in cell-free extracts. The results presented in Table V bring out certain significant facts. In the presence of the iron-binding fraction, Fe^{59} incorporation into protoporphyrin is significantly enhanced as compared to when $Fe^{59}SO_4$ alone is used. The results presented in Table VI indicate that intact XFe^{59} (isolated from cobalt-toxic or iron deficient culture fluid) can act as a better iron source than $Fe^{59}SO_4$ emphasising the superiority of this type of organic iron over $Fe^{59}SO_4$, as an iron source for heme synthesis in cell-free extracts. It is interesting to note that when the organic iron is used as the iron source, the non-enzymic incorporation into protoporphyrin is quite high and is of the same order as that of enzymic incorporation of inorganic iron. Cobalt inhibits (Table V) iron incorporation into protoporphyrin even in presence of the iron-binding fraction when present in definite excess of iron. However, when cobalt is used in amounts equivalent to and in place of iron, its incorporation is less than 10% that of iron.

Table III. Effect of cobalt on Catalase, Iron-binding Compound production and Heme - Fe^{59} in the mycelia as a function of growth period in N. crassa

Fe^{59} was included to give 6.89×10^{4} counts/min/10 ml basal medium. The experimental details are given in text.

Period of growth hr	Normal				Cobalt-toxic			
	growth mg dry wt	Catalase 0.01M $KMnO_4$/mg protein/ 5 min	Iron-binding compound O.D. at 440 mμ	Heme Fe^{59} cpm/10 mg hemin/ 100 mg dry wt	growth mg dry wt	Catalase 0.01M $KMnO_4$/mg protein/ 5 min	Iron-binding compound O.D. at 440 mμ	Heme Fe^{59} cpm/10 mg hemin/ 100 mg dry wt
24	8.2	7.2	-	285	3.2	8.5	0.04	311
48	37.0	39.2	-	351	19.0	10.4	0.12	283
72	46.2	52.3	0.03	423	25.2	11.0	0.22	255

Table IV. Effect of cobalt on glycine-2-C^{14} incorporation into the mycelial heme fraction of *N. crassa*.

Glycine-2-C^{14} was included to give 1.33×10^4 counts/min/10 ml basal medium containing 48 µg glycine. The mycelia were processed after 72 hr. growth.

The experimental details are given in text.

Treatment	Total incorporation counts/min/ g. dry weight $\times 10^5$	Incorporation into heme fraction. counts/min/ 10 mg hemin/ g dry wt.
Normal	2.50	143
Cobalt-toxic	3.55	97

Table V. Effect of cobalt on Fe^{59} incorporation into protoporphyrin in cell-free extracts of N. crassa

The incubation mixture in 2 ml contained: Iron ($Fe^{59}SO_4$)- 24 mμmoles cobalt ($Co^{60}Cl_2$) - 30 mμmoles; Protoporphyrin - 30 mμmoles; Tris (pH 7.8) - 180 μmoles; Ascorbic acid - 40 μ moles; Enzyme - 0.5 ml containing 6 mg protein/ml. The iron binding fraction prepared from XFe was added in 0.1 ml and in slight excess equivalents of the iron present in the incubation mixture. Fe^{59} and Co^{60} were included to give 1.42×10^5 counts/min.

The experimental details are given in text.

Metal source	Radioactivity in hemin counts/min/10 mg hemin
$Fe^{59}SO_4$	516
$Fe^{59}SO_4$*	50
$Fe^{59}SO_4$ + 30 mμmoles Co	455
$Fe^{59}SO_4$ + 60 mμmoles Co	432
$Fe^{59}SO_4$ + 150 mμmoles Co	313
$Fe^{59}SO_4$ + Iron-binding fraction	1521
$Fe^{59}SO_4$ + Iron-binding fraction*	580
$Fe^{59}SO4$ + Iron-binding fraction + 30 mμmoles Co	1310
$Fe^{59}SO_4$ + Iron-binding fraction + 60 mμmoles Co	1251
$Fe^{59}SO_4$ + Iron-binding fraction + 150 mμmoles Co	773
$Co^{60}Cl_2$	45

* non-enzymic incorporation (enzyme denatured or omitted)

Table VI. Iron incorporation from XFe^{59} and $Fe^{59}SO_4$ into protoporphyrin in cell-free extracts of Neurospora crassa

The low specific activity of the isolated XFe^{59} necessitated the use of high levels of substrates. The incubation mixture in 5 ml contained: Iron ($Fe^{59}SO_4$ or XFe^{59}) - 0.5 µmole; Protoporphyrin - 0.6 µmole; Tris (pH 7.8) 360 µ moles; Ascorbic acid - 80 µmoles; Enzyme - 2 ml containing 6 mg protein/ml. The radioactivity included gave 3.71×10^4 counts/min.

The experimental details are given in text.

Iron source	Radioactivity in hemin counts/min/10 mg hemin
$Fe^{59}SO_4$	900
$Fe^{59}SO_4$*	98
XFe^{59}	1756
XFe^{59}*	797

* non-enzymic incorporation (enzyme denatured or omitted)

DISCUSSION

Increasing concentrations of cobalt result in increased production of the iron-binding compound and a corresponding fall in catalase activity (Table I). An identical picture has been obtained when there is a decrease in the iron concentration of the medium from the optimal level (Chapter II). This is clearly indicative of a conditioned iron deficiency brought about by cobalt. This conditioned iron deficiency is not brought about by an inhibition of iron uptake by the organism due to cobalt. Instead, the effect of cobalt is to accelerate the iron uptake and deplete the medium of iron at an early stage of growth. However, there is a significant decrease in bound Fe^{59} in presence of cobalt at 72 hr of growth (Table II) indicating that cobalt interferes with the utilization of iron for the formation of key metabolites. Similar results have been obtained by Tor-Magnus Ehari (9), who has found in the yeast Candida guilliermondii that cobalt enhances iron uptake by the organism but there is a decrease in the trichloroacetic precipitable iron and the iron incorporated into the particulate and supernatant fractions of the yeast under these conditions.

The metabolic potency of XFe as an iron donor to N. crassa and the rapid incorporation of XFe^{59} as compared to Fe^{59}-citrate or $Fe^{59}Cl_3$ into the mycelia have been taken to explain the survival value of the phenomenon of iron-binding compound secretion under conditions of straight iron deficiency or cobalt toxicity. Further, it has been found that N. crassa elaborates organic iron chelates very similar to XFe under normal conditions when grown with optimal levels of iron and the possibility has been envisaged that this type of iron chelate may play a key role in iron transport in this organism (Chapter II). In this context, it is significant that the primary event of induced iron deficiency appears to be

the secretion of the iron-binding compound even before catalase activity, bound Fe^{59} and heme Fe^{59} show a decrease. Rothstein and Hayes (10) have concluded from experiments carried out in yeast, that the cation binding sites are undoubtedly located at the periphery of the cell. Thus, the rapid depletion of iron from the medium in cobalt toxicity can leave the cell surface in contact with the iron depleted medium. The unavailability of iron for the formation of the natural iron chelate results in the secretion of the iron-binding compound prior to affecting the other iron-dependent systems. Similar results have been obtained when the organism is grown under conditions of straight iron deficiency (Chapter II). The mechanism of the secretion of the iron-binding compound in large amounts in iron deficiency is not clear and some of the possibilities have been discussed by Neilands (11). It may be noted that at 24 hr of growth in presence of cobalt, when iron deficiency may be considered to have just set in, the growth of the organism is significantly inhibited (Table III). This may represent a direct toxic effect of cobalt not involving a conditioned iron deficiency.

The intracellular cobalt-iron antagonism is evident at the level of heme synthesis. Here, the effects of cobalt are two fold. It inhibits the synthesis of the porphyrin moiety and also the iron incorporation into protoporphyrin in cell-free extracts. Cobalt has been found to inhibit incorporation of radioactive glycine into heme by preparations of rabbit bone marrow (12).

The metabolic potency of XFe^{59} as a better iron source than $Fe^{59}SO_4$ for heme synthesis in cell-free extracts (Table VI) emphazises the possibility that this type of organic iron formed in the cell under normal conditions can act as an iron donor for heme synthesis in vivo.

The fact that the iron-binding fraction, which has poor binding affinity for Fe^{++} can enhance iron ($FeSO_4$) incorporation into protoporphyrin (Table V) lends support to the suggestion that the Fe^{+++} once coordinated as the trihydroxamate can be transported to or into the cell and donated to the iron enzymes which may involve a one-electron reduction. It is also held that the reduction and release of iron from the hydroxamte can be most profitable only when these processes operate at the site of incorporation into the iron-containing enzymes and prothetic groups (13). The possibility has been envisaged that at least in microorganisms, the sideramines may play an important part in the enzymatic incorporation of iron into porphyrins (14). Cobalt inhibits iron incorporation into protoporphyrin even in presence of the iron-binding fraction, when present in definite excess of iron (Table V). This indicates that (a) subsequent to the depletion of iron in the medium, a high intracellualr concentration of cobalt has to be built up to result in decreased iron incorporation and heme synthesis and (b) cobalt may not only render iron unavialable for the formation of the natural organic iron chelate but also interfere with the iron utilization from the chelate formed under conditions of normal iron supply.

Another interesting feature of the results presented in Table V is the poor incorporation of Co^{60} into protoporphyrin even though it inhibits iron incorporation. Labbe and Hubbard (7) have shown that the rat liver iron-protoporphyrin chelating enzyme can utilize both iron and cobalt for the respective heme formation with equal facility. But the behaviour of cobalt in N. crassa is similar to that of manganese in the rat liver system, which though inhibits iron incorporation into protoporphyrin does not itself get incorporated to an appreciable extent. The chelating enzyme from chicken erythrocytes has been shown to utilize Co^{60} to the

extent of only 2% of the Fe^{59} incorporated (15), although Johnson and Jones (16) attribute this to the use of ethyl acetate for the extraction of the metalloporphyrins ~~wi~~ in which cobalto-porphyrin has been found to be sparingly soluble.

Attempts to isolate Co^{60} heme from the cobalt-toxic mycelia of N. crassa have not been successful. This can be attributed to an inhibited heme synthesis and to the poor incorporation of Co^{60} into protoporphyrin even though cobalt inhibits iron incorporation and finally to a dilution of the label as a result of the excess cobalt used to produce toxicity.

SUMMARY

1. Increasing concentrations of cobalt result in increasing production of the iron-binding compound (X) and a corresponding fall in catalase activity in N. crassa

2. Cobalt accelerates iron uptake by the organism and depletes the medium of iron at an early stage of growth.

3. The production of the iron-binding compound (X) precedes the fall in catalase activity, bound Fe^{59} and heme Fe^{59} values under cobalt toxic conditions.

4. XFe^{59} acts as better iron source as compared to $Fe^{59}SO_4$ for heme synthesis in cell-free extracts of N. crassa.

5. Cobalt inhibits synthesis of the porphyrin moiety. It also inhibits iron incorporation into protoporphyrin significantly, when present in definite excess of iron. Cobalt itself does not get incorporated into protoporphyrin to an appreciable extent.

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CHAPTER IV

STUDIES ON COBALT AND NICKEL TOXICITIES IN *NEUROSPORA CRASSA*

STUDIES ON COBALT AND NICKEL TOXICITIES IN *NEUROSPORA CRASSA*

Earlier studies by Adiga, Sivaramasastry, Venkatasubramanyam and Sarma (1) on the toxicities caused by the heavy metals cobalt, nickel and zinc in *Aspergillus niger* and subsequent studies by the same authors in *Neurospora crassa* (2), *Corcyra cephalonica* st (3) and germinating seedlings of *Phaseolus radiatus* (4) have revealed a complex effect on growth and metabolism. Generally, iron and magnesium have been found to counteract growth inhibition due to the toxic metals. However, the nature and magnitude of the derangements induced are dependent on the type of the heavy metal and the nature of the organism in question.

In *Aspergillus niger* while a uniformly high level of iron is required to counteract growth inhibition due to the three heavy metals, the magnesium concentration required for the same differs markedly with the particular heavy metal employed. Further, iron can reverse impaired glucose utilization only in cobalt toxicity but not in nickel and zinc toxicities (1). Studies by Healy, Cheng and McElroy (5) and Sivaramasastry *et al* (2) in *Neurospora crassa* as well as the subsequent detection of an iron-binding compound in the culture fluid of the same organism under conditions of cobalt toxicity or straight iron deficiency (Chapter I) clearly emphasize a direct cobalt-iron antagonism in this organism. Presumably the effects of nickel and zinc are indirect so far as iron metabolism is concerned since iron is able to counteract the growth inhibition due to all the three heavy metal toxicities in *Neurospora crassa* as well. Further, it has been found that magnesium counteracts growth inhibition due to the heavy metals by interfering with the uptake of the toxic metal, where as iron exerts a beneficial effect without

the net accumulation of the toxic metal inside the mycelium (2,6). These studies imply not only a difference in the modes of counteraction by iron and magnesium but also that the heavy metals themselves may differ in their sites of toxic action. Thus, the toxic metals may be affecting key metabolic processes and the levels of vital metabolites in dissimilar ways, though leading to an inhibition of overall growth to the same extent.

In this chapter, the results obtained on the influence of cobalt and nickel on (i) the mycelial protein status (ii) the activities of a few degradative enzymes and (iii) the metabolism of carbohydrate intermediates, in N. crassa are presented.

EXPERIMENTAL

Materials.

Co^{60} (carrier-free) was purchased from the Radiochemical Centre, Amersham, U.K. Citric acid (Merck), Succinic acid (BDH), L-malic acid and Fumaric acid (NBC) and Pyruvic acid (sodium salt, Sigma) were pure commercial preparations. β-glycerol-phosphate (sodium salt)was of analytical grade. Crystalline hemoglobin was purchased from Nutritional Biochemical Corporation.

Organism and culture conditions

Neurospora crassa Em 5297a (wild) was used in these studies. The culture and growth conditions have been described in Chapter I.

Effect of cobalt and nickel on growth and protein content.

Cobalt and nickel were included at 800 µg and 500 µg per 10 ml basal medium respectively to produce around 50% growth inhibition at the end of 72 hr growth in stationary culture. The mycelia were removed at

different intervals of time, washed and the weights recorded after drying in an oven at 60°. overnight. The mycelia were then processed to estimate the TCA precipitable protein as described in the following section.

Effect of cobalt and nickel on $S^{35}O_4$ incorporation into protein in preformed mycelia.

The influence of the toxic metals on protein synthesis was studied in preformed normal mycelia. N. crassa was grown for 40 hr in 10 ml basal medium. The 40 hr old normal mycelium, in the middle of the log phase growth, was washed and reincubated in 5 ml of fresh medium in 50 ml flasks containing different levels of the toxic metal. The composition of this medium was the same as described in chapter I except that $MgSO_4.7\ H_2O$ was replaced by $MgCl_2.7H_2O$ and an amount of sulphur equivalent to 50 µg was provided as Na_2SO_4. Radioactive sulphur was provided as carrier-free

The flasks were shaken in a reciprocal shaker and the mycelia were removed at different intervals of time. The mycelia were washed to free of adhering radioactivity and after recording the fresh weights, were processed to determine the total and protein sulphur incorporation.

Each mycelium was ground with 4 ml of water and an aliquot of the fine suspension was plancheted on to stainless steel planchets to measure total suphur incorporation. The rest of the sample was precipitated with cold TCA to give a final concentration of 10% and centrifuged. The precipitate was washed thrice with cold 10% TCA and the supernatants were pooled. The cold TCA precipitate was then washed twice with alcohol:ether (3:1), treating the precipitate each time with 4 ml of the reagent for 10 min. at 90°. This was followed by two treatments with hot 10% TCA.

using 4 ml each time. The precipitate was finally washed with ether to remove the TCA and suspended in 4 ml of water. A fine suspension was made thereafter and aliquots were used to measure radioactivity and protein content.

The pooled cold 10% TCA supernatant was treated with ether to remove the TCA. After adding carrier sulphate, aliquots were treated with Ba^{++} to remove sulphate and the precipitated $BaS^{35}O_4$ was washed twice and counted. The S^{35} in the supernatant after $BaS^{35}O_4$ precipitation was taken to represent the radioactivity in the free amino acid pool.

Radioactivity measurements were carried out with a Geiger-Muller end window counter attached to a decade scaler (type 151A, Nuclear Chicago Corporation, Des Plaines, Illinois, U.S.A.). The error due to count rate was within $\pm$ 2%. Appropriate self-absorption, background and decay corrections were applied.

Protein content was measured by the method of Lowry et al (7).

Acid and alkaline phosphatases

The effects of cobalt and nickel on these enzyme levels were studied by growing the organism with different levels of the toxic metal for 72 hr and then assaying for the activities of these enzymes in the toxic mycelia.

The enzyme extract was prepared by grinding the fresh mycelia with glass powder and Tris buffer (pH 7.0, 0.05 M) using a pestle and mortar. The extract was centrifuged at 3000 x g for 15 min and the

precipitate was washed once with the buffer. The supernatants were pooled and used as the enzyme source. All the operations were carried

The enzymes were assayed according to the method of Kuo and Blumenthol (8,9) who have purified the acid and alkaline phosphatases from the same strain of N. crassa used in the present study. Acid phosphatase was assayed at its optimum pH of 5.6. The incubation mixture in a total volume of 1.5 ml contained: sodium β-glycerol phosphate (pH 5.6) - 40 μ moles; acetate buffer (pH 5.6) - 100 μ moles; enzyme - 0.5 ml. The mixture was incubated at 30° for 20 min and the reaction was arrested with 0.5 ml of 50% TCA. The denatured protein was centrifuged out and the liberated inorganic phosphorus was estimated in the supernatant. The enzyme activity is expressed as μg P liberated/mg protein/20 min. Alkaline Phosphatase was assayed at pH 8.9 in presence of 20 μ moles of $MgSO_4 \cdot 7H_2O$.

Estimation of phosphorus (10)

To the TCA supernatant containing 0.1 - 1.0 μmole of phosphorus in 1.0 ml, 1.0 ml of 5N sulphuric acid was added followed by 1 ml of 2.5% ammonium molybdate. After mixing, 0.1 ml of the reducing reagent was added. (The reducing reagent was prepared in powdered form and dissolved before use. 0.2 g of 1-amino-2-naphthol-4 sulfonic acid was mixed with 1.2 g of sodium bisulphite and 1.2 g of sodium sulphite. Before use, 0.25 g was dissolved in 10.0 ml of water). The volume was made upto 10 ml and after mixing the absorbance was measured in a Klett-Summerson colorimeter with the filter No. 66.

Proteinase

The enzyme extract prepared as indicated earlier was used to assay proteinase activity using hemoglobin as substrate. It was found that when the enzyme solution was incubated at the optimum pH of 4.2 (fig IV) at 37°, precipitation ensued but the activity was found to reside in the

supernatant. So, the supernatant obtained after centrifuging the crude extract incubated at pH 4.2 at 37° for 10 min was used as the enzyme source. 1.0 ml of this enzyme preparation was incubated with 0.5 ml of 1% hemoglobin and 1.0 ml of citrate-phosphate buffer (pH 4.2) for 30 min at 37°. The reaction was stopped with 0.5 ml of 50% TCA and centrifuged. Aliquots of the supernatant were used for the estimation of tyrosine by the method of Lowry et al (7). This method has been described in detail in Chapter II. The enzyme activity is expressed as µg tyrosine/mg protein/

of Krebs' cycle intermediates on cobalt and nickel toxicities

The effect of Kreb's Cycle intermediates on growth inhibition due to the heavy metals was studied by including them along with the toxic metal and assessing the mycelial dry weight at the end of 72 hr growth. These intermediates were included at levels at which their counteracting effects were maximal. Citrate, pyruvate, α-ketoglutarate, succinate and malate were added at a level of 200 mg/10 ml basal medium. Fumarate was included at 70 mg/10 ml, since higher concentrations could not be tried due to its relative insolubility. The organic acids were included as aqueous solutions (pH 4.8) along with the toxic metal (800 µg cobalt or 500 µg nickel) in the medium. Pyruvate and α-ketoglutarate were sterilized in the cold by passing through a sterilized Seitz filter fitted with an asbestos pad, autoclaved previously, and added aseptically to the rest of the medium which was separately autoclaved at 15 lbs/sq in. steam pressure for 10 minutes.

Incorporation of cobalt and nickel into the

The effect of the carbohydrate intermediates on the uptake of

the heavy metals cobalt and nickel were also studied in the above experiments. Carrier-free $Co^{60}Cl_2$ was included along with 800 µg cobalt and the organic acid in 10 ml of the basal medium and at the end of 72 hr growth, the mycelia were washed free of adhering radioactivity and then dried in an oven at 60° overnight. After recording the dry weights, the mycelia were digested with acid and the acid digests were used to measure radioactivity in a scintillation detector attached to a decade scaler (type 151A, Nuclear Chicago Corporation, Illinois, U.S.A.). From the amount of radioactivity recovered in the mycelium, the amount of cobalt incorporated into the mycelium was calculated.

In experiments on the uptake of nickel, the mycelial acid digests were used for the chemical estimation of nickel using dimethyl glyoxime, citrate being added to avoid interference due to iron (11). The mycelia were subjected to a wet-ashing rpocedure using HNO_3:$HClO_4$ (10:1) mixture. The residue was taken in a known volume of 1N HCl. A 3.2 ml aliquot was treated in a test tube with 0.2 ml of saturated bromine-water and the oxidation allowed to proceed for 5 minutes. 1.0 ml of 10% aqueous solution of sodium citrate was rapidly added, immediately followed by 0.4 ml of liquor ammonia (sp gr. 0.8). and the contents were shaken vigorously for 2 minutes. 2.0 ml of ethyl alcohol (95%) was then added followed by 0.2 ml of 1%(w/v) dimethyl glyoxime reagent in 95% ethanol. The contents of the tube were shaken well and the red color developed was measured against an appropriate blank in a Klett-Summerson colorimeter using filter No. 49.

The effect of iron and magnesium on the uptake of cobalt and nickel were also studied in stationary growth culture experiments by procedures described above. Iron or magnesium was included at 2000 µg/10 ml basal medium along with the respective toxic metal, 800 µg cobalt or 500 µg

nickel. The mycelia were removed at different intervals of growth period and the cobalt and nickel contents were estiamted by methods already described.

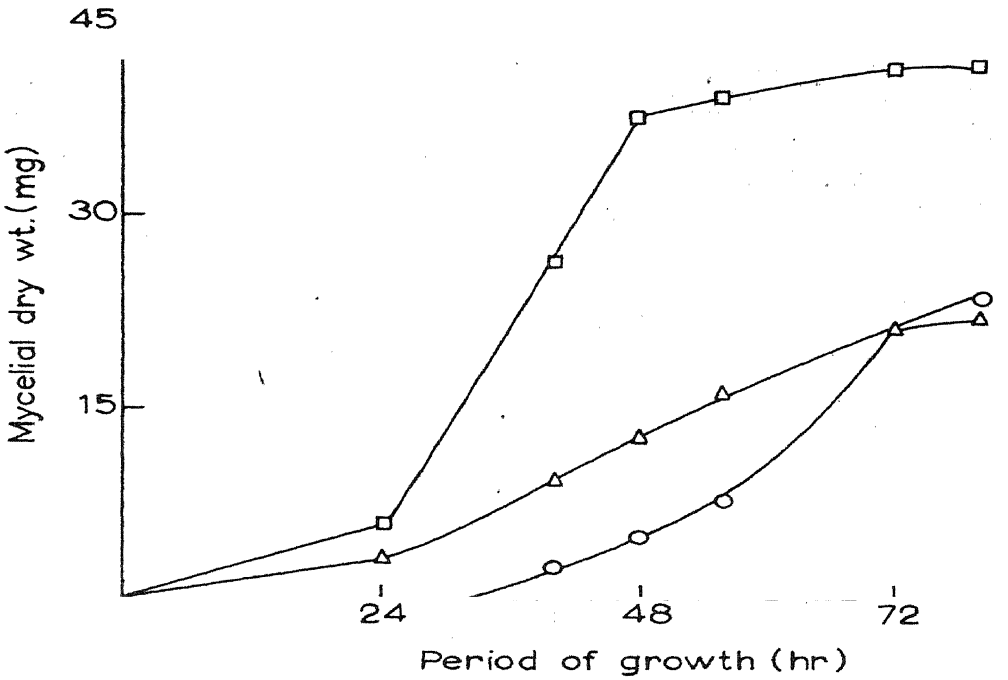
RESULTS

Fig. I and II indicate changes in dry weight and mycelial protein concentration of M. crassa as a function of the growth period, when the organism is grown under cobalt and nickel toxic conditions. It can be seen that the primary effect of nickel is a delay in the initiation of mycelial growth, which starts only 36 hr after inoculation. Thereafter nickel toxic mycelia grow at a rapid rate and at the end of 72 hr. both cobalt and nickel toxic mycelia reach the same weights (Fig. I). Strikingly enough, the protein concentration of the nickel toxic mycelium is significantly higher as compared to the normal or cobalt-toxic mycelia through out the period of noticeable growth (fig. II).

The net protein status of the mycelium is determined by several factors such as the effect of the heavy metals on mycelial protein synthesis, non-protein material synthesis as well as the effects on their degradation. A study of the influence of the toxic metals on S^{35} incorporation into preformed log phase normal mycelia indicates that neither metal has any effect on sulphur incorporation into the mycelial protein at concentrations

can induce 50% growth inhibition (400 µg cobalt and 250 µg nickel per 5 ml medium) in stationary culture at the end of 72 hours. But certain differences in the action of the heavy metals are discernable at high concentrations. Cobalt which has no effect on total sulphur incorporation into the mycelia tends to inhibit the percent of the total incorporated into the protein with a corresponding increase in that incorporated into the free amino acid pool. Fig. III indicates the effect of 800 µg

Effect of cobalt and nickel on the growth of N.crassa



□ Normal

△ Cobalt (800 µg/10 ml medium)

○ Nickel (500 µg/10 ml medium)

Fig.II

Effect of cobalt and nickel on protein ation of the mycelia of *N. crassa*.

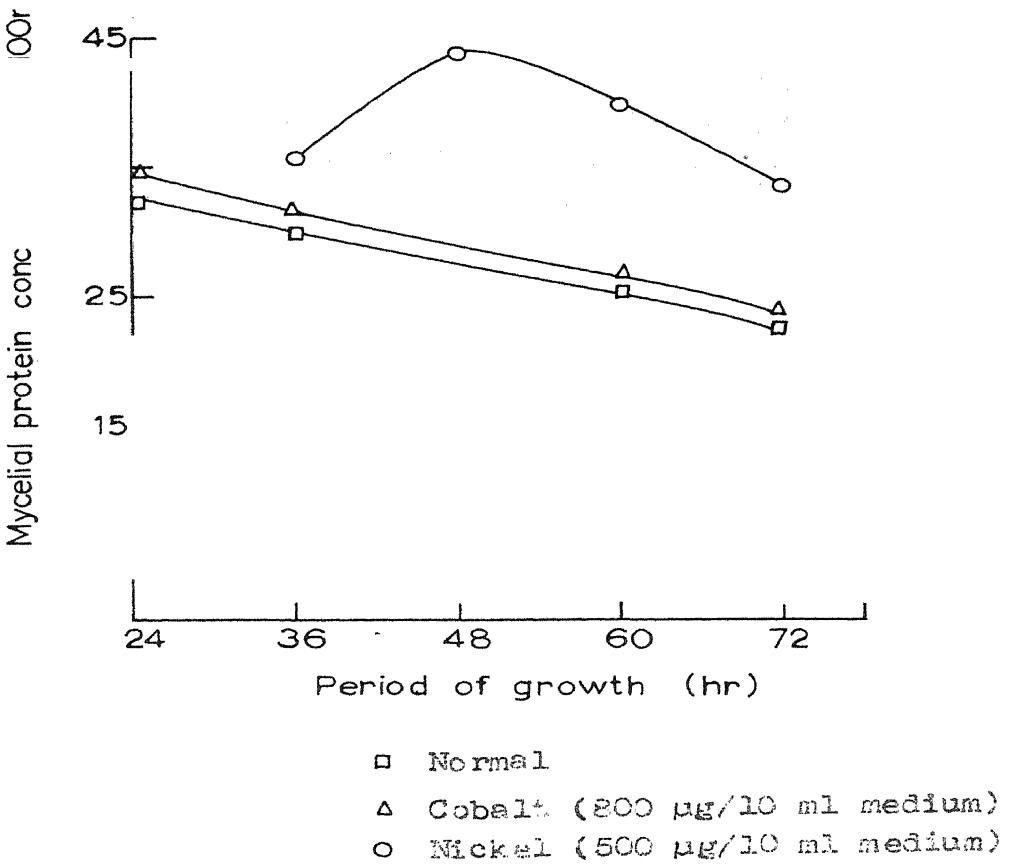
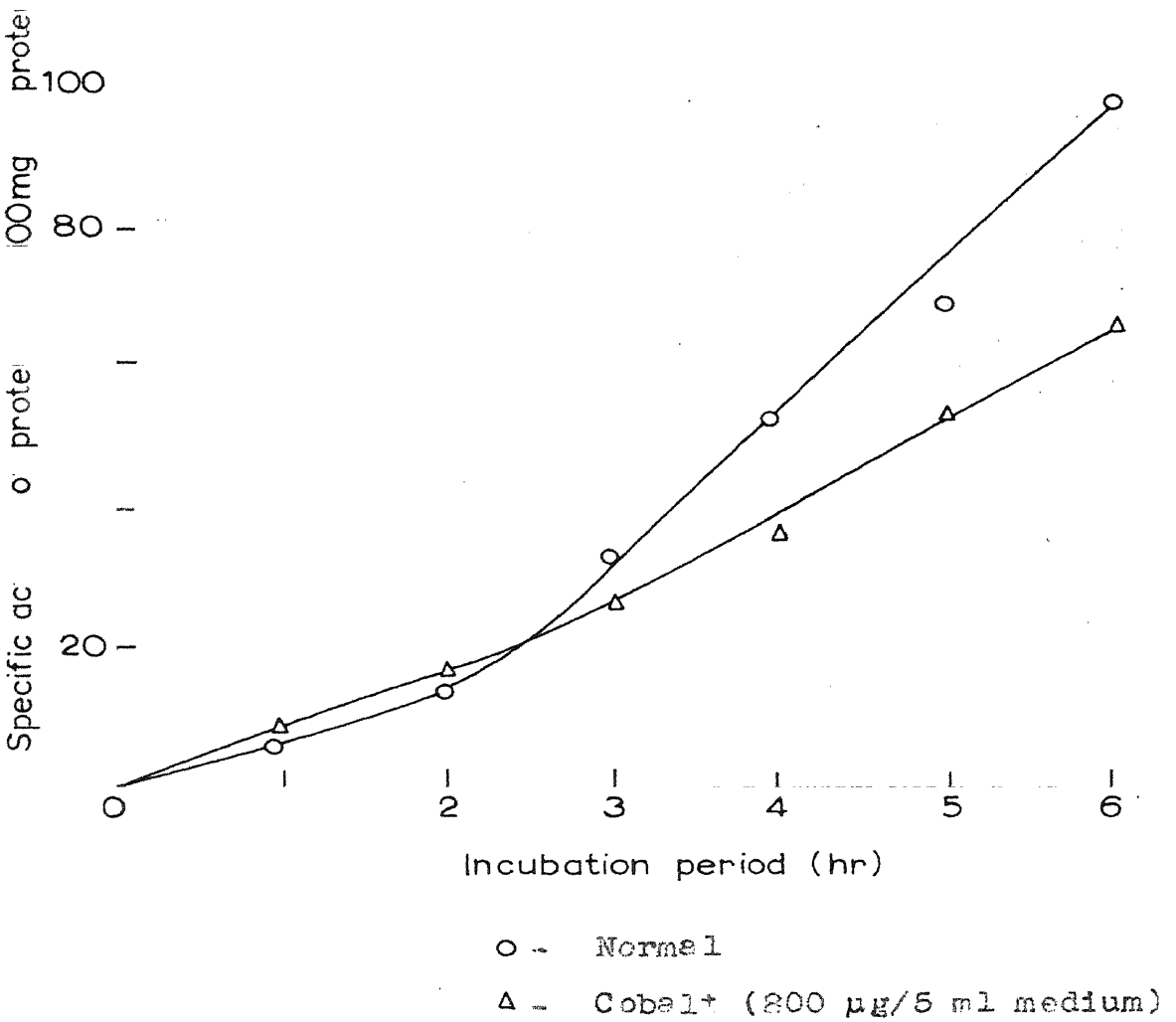


Fig.III

Effect [illegible] cobalt on the specific activity of the mycelial [illegible]



cobalt/5 ml medium on the specific activity of the mycelial protein as a function of the incubation time Nickel tends to inhibit total sulphur incorporation into the mycelia, but the percent of the total incorporated into the protein shows an increase with an increase in the concentration of heavy metal (Table I).

Interesting results have been obtained on the effects of cobalt and nickel on the levels of acid and alkaline phosphatases and proteinase. The proteinase enzyme from N. crassa has been found to have a pH optimum of 4.2 with hemoglobin as substrate (fig. IV). Fig. VI indicates that the proteinas and the phosphatases show a striking increase when the organism is grown with increasing levels of cobalt. However, increasing levels of nickel do not appreciably affect the enzyme activities. Different concentrations of cobalt and nickel neither activate nor inhibit these enzyme activities in vitro as is evident from the results presented in Table II.

Significant differences can also be observed in the responses of the two heavy metal toxicities to the TCA cycle intermediates. Citrate and malate have been found to annul growth inhibition due to cobalt as well as nickel toxicities. Pyruvate and α-ketoglutarate are effective in nickel toxicity but not in the case of cobalt. Fumarate and succinate do not have a beneficial influence in either case and the presence of succinate along with the heavy metal results in added inhibition of growth. Citrate and malate inhibit the incorporation of the heavy metals into the mycelia. The keto acids strikingly enhance the uptake of cobalt. They do not have any effect on the uptake of nickel. Succinate and fumarate also have an enhancing effect on cobalt incorporation into the mycelia. These results are summarised in Table III.

Table I. Effect of increasing concentrations of cobalt and nickel on sulphur incorporation into preformed normal mycelia of N. crassa

Carrier-free $Na_2S^{35}O_4$ was included along with 50 µg sulphur in 5.0 ml incubating medium. Incubation period, 6 hr. The results are expressed in terms of µg sulphur incorporated.

The experimental details are given in text.

Metal (µg/5 ml)	Total sulphur incorporated (µg S/100 mg fresh wt)	Percent sulphur of total incorporated into		
		Sulphate	Amino acids	Protein
None (Control)	6.2	4.1	30.5	39.7
Cobalt (400)	6.4	4.0	32.0	37.7
Cobalt (800)	6.1	3.4	35.0	33.2
Cobalt (1200)	6.5	3.3	39.3	29.1
Cobalt (1600)	6.2	3.0	43.7	24.6
Nickel (250)	6.2	4.0	29.9	41.0
Nickel (500)	5.5	4.2	27.5	43.2
Nickel (750)	4.9	4.1	25.7	46.9
Nickel (1000)	4.2	4.0	24.0	49.0

Fig.IV

pH optimum of proteinase from *N. crassa*

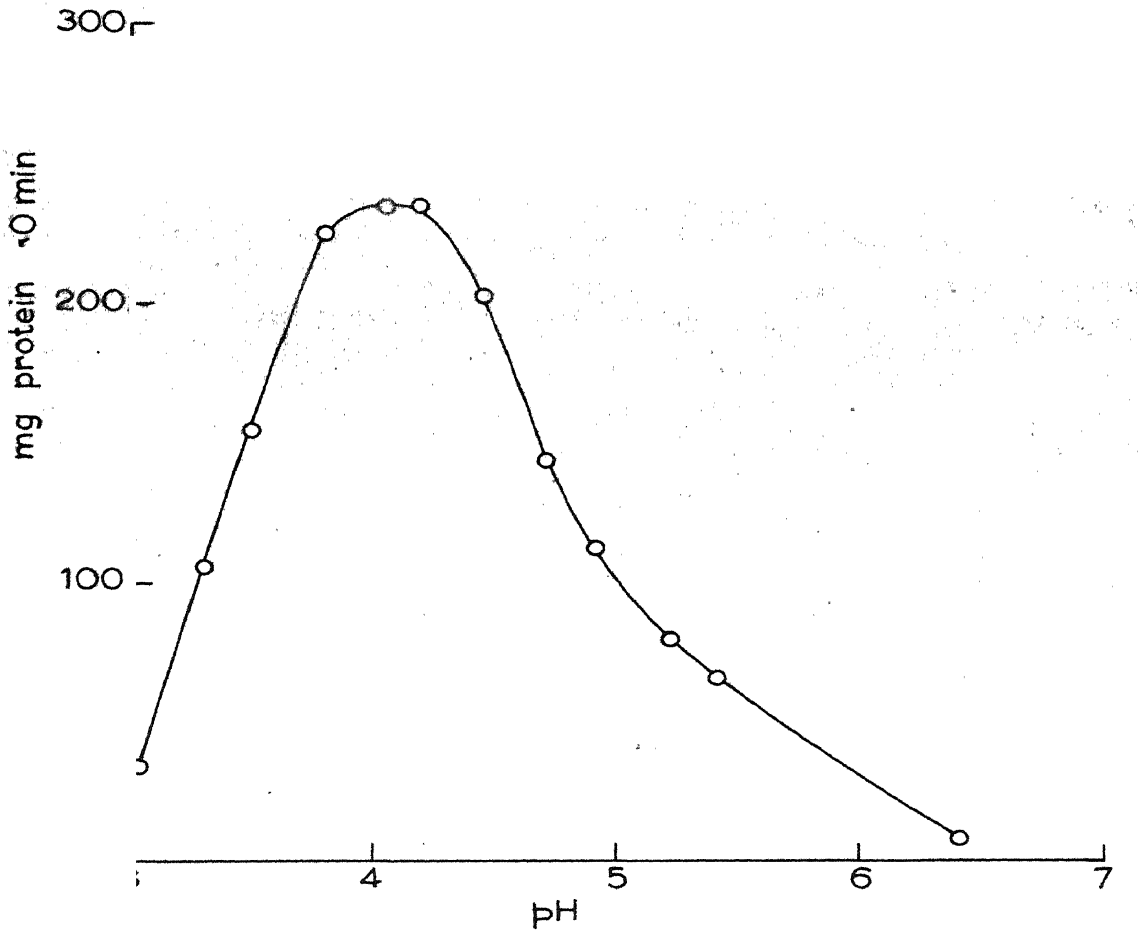


Table II. Effect of cobalt and nickel on proteinase, acid and alkaline phosphatases

The enzyme extract from 72 hr old normal mycelia were preincubated with the metal for 10 min and then assayed for activity.

The experimental details are given in text.

Metal	Proteinase µg tyrosine/ mg protein 30 min	Acid phosphatase µg phosphorus/mg protein 20 min	Alkaline phosphatas µg phosphorus/mg protein 20 min
None	212.1	30.4	41.6
$CoCl_2 . 6 H_2O$			
1×10^{-6} M	232.1	32.4	45.2
1×10^{-5} M	222.1	28.4	40.3
1×10^{-4} M	214.2	29.2	42.3
$NiSO_4 . 6 H_2O$			
1×10^{-6} M	218.1	29.6	45.6
1×10^{-5} M	220.1	34.1	42.6
1×10^{-4} M	209.2	30.7	41.5

Table III. Effect of TCA cycle intermediates on cobalt and nickel toxicities in *N. crassa*.

Supplements to the basal medium	Mycelial dry wt (mg)	Metal uptake µg/100 mg dry mycelium
None (Control)	43.0	-
800 µg cobalt	23.0	33.1
800 µg cobalt + 200 mg citrate	40.0	7.0
800 µg cobalt + 200 mg pyruvate	26.6	124.0
800 µg cobalt + 200 mg α-ketoglutarate	25.1	69.4
800 µg cobalt + 200 mg succinate	5.0	48.1
800 µg cobalt + 70 mg fumarate	23.1	44.1
800 µg cobalt + 200 mg malate	43.6	19.0
500 µg Nickel	24.2	12.1
500 µg nickel + 200 mg citrate	44.2	2.1
500 µg nickel + 200 mg pyruvate	39.2	13.1
500 µg nickel + 200 mg α-ketoglutarate	40.1	14.2
500 µg nickel + 200 mg succinate	10.2	12.6
500 µg nickel + 70 mg fumarate	20.1	11.9
500 µg nickel + 200 mg malate	48.0	5.9
200 mg citrate	46.4	-
200 mg pyruvate	42.0	-
200 mg α-ketoglutarate	43.6	-
200 mg succinate	43.0	-
70 mg fumarate	45.0	-
200 mg malate	48.6	-

Fig. V

Proteinase activity of *N. crassa* as a function of incubation period.

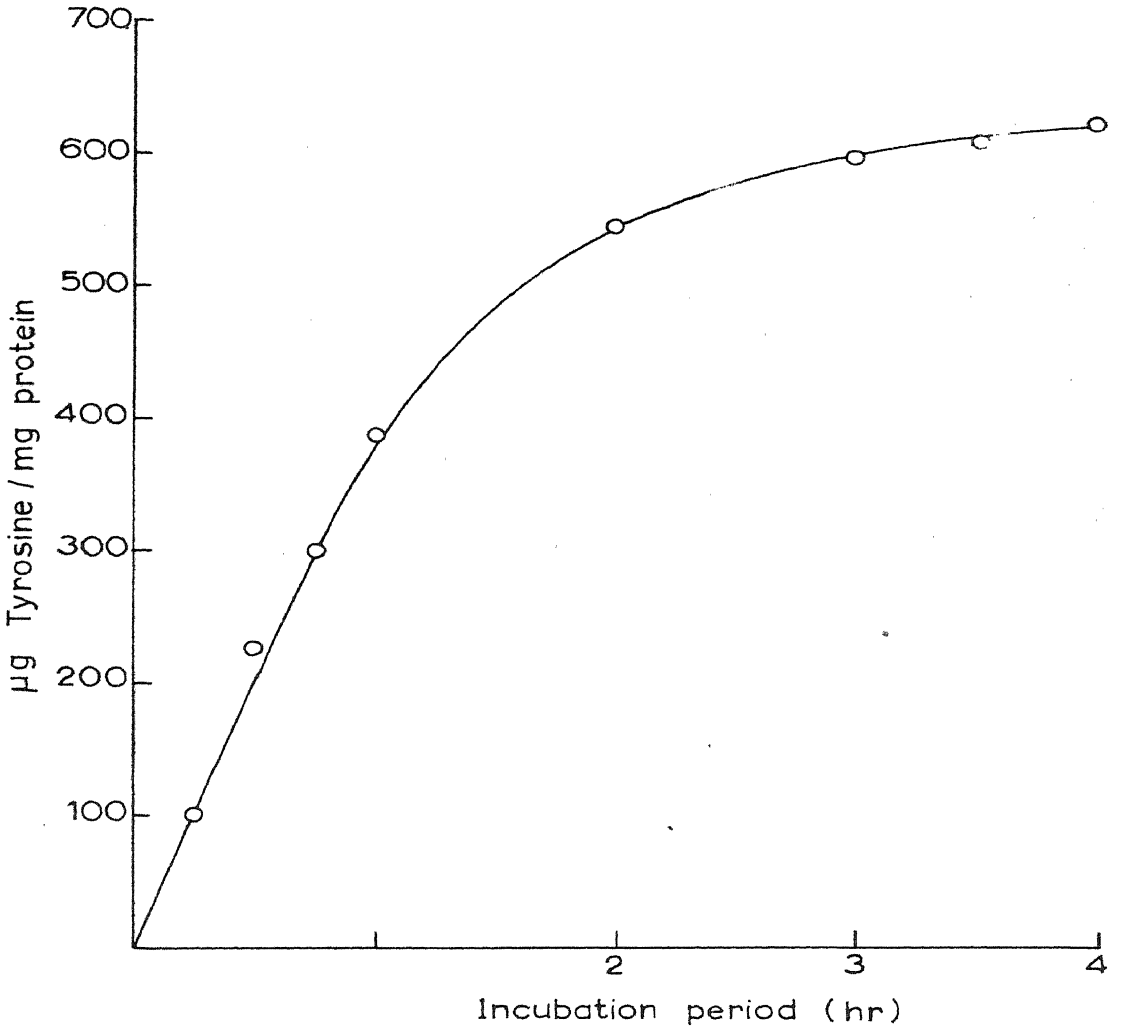
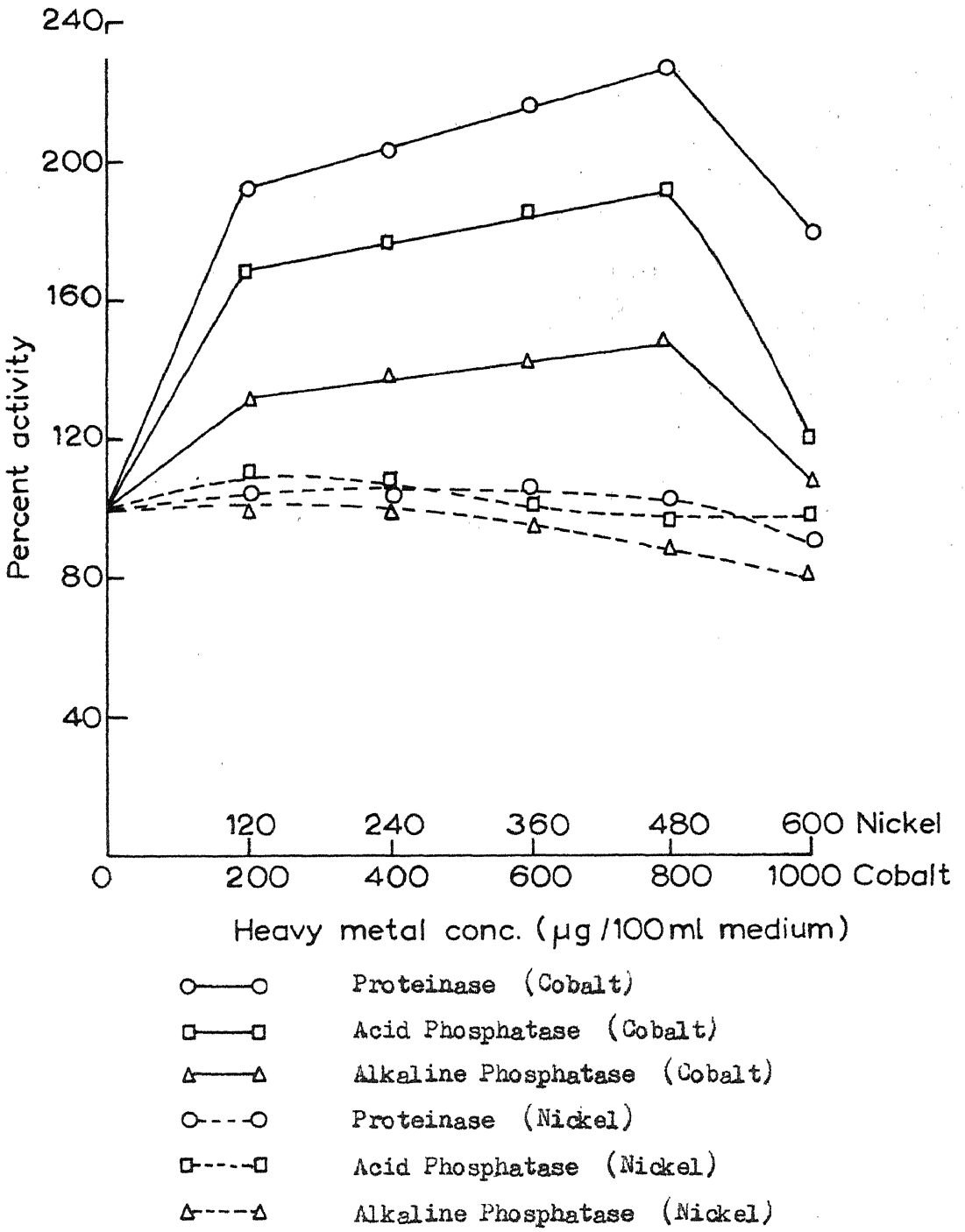
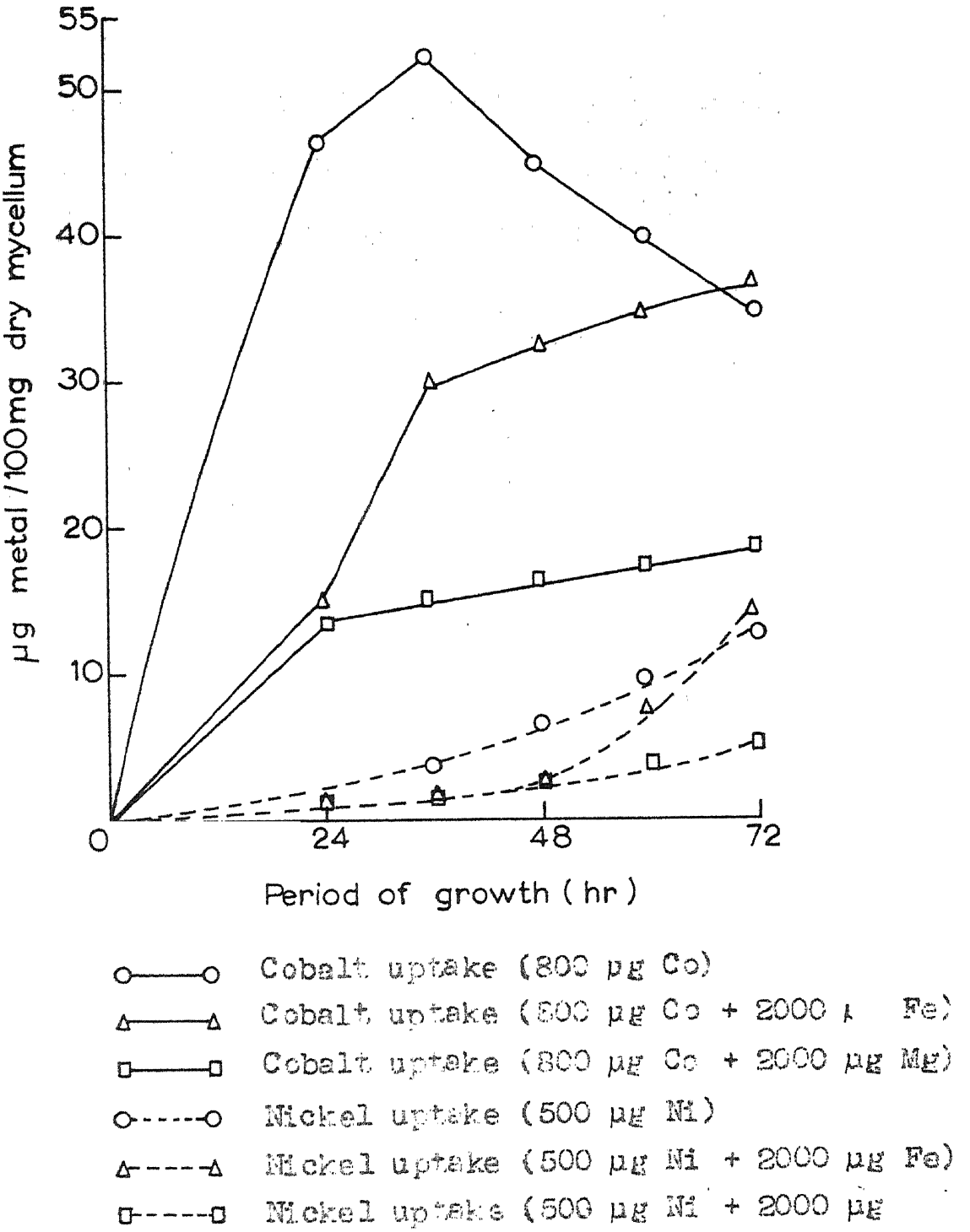


FIG. VI

EFFECT OF COBALT AND NICKEL ON PROTEINASE, ACID AND PHOSPHATASES OF N.C



Effect of iron and magnesium on the uptake of cobalt and nickel by N. crassa as a function of the g period.



The effects of iron and magnesium on the uptake of cobalt and nickel by the mycelia grown in presence of the toxic and counteracting metals are depicted in fig VII. It can be seen that both iron and magnesium suppress incorporation of the toxic metals at 24 hr of growth. Thereafter, magnesium maintains its inhibitory influence on toxic metal uptake, but that due to iron is released. At the end of 72 hr growth, it appears as if iron has brought about a reversal of growth inhibition without affecting the net accumulation of the toxic metal inside the mycelium, as has been observed earlier by Sivaramasastry et al (2)

DISCUSSION

These investigations reveal certain differences in the sites of toxic action of closely related elements like cobalt and nickel at concentrations leading to an inhibition of overall growth to the same extent. A primary effect of nickel seems to be a delay in the initiation of mycelial growth (fig. I). Nickel toxic mycelia contain significantly higher concentrations of protein as compared to the normal or cobalt-toxic mycelia (fig. II). In preformed normal log phase mycelia neither cobalt nor nickel show a specific inhibition of protein synthesis at concentrations which can induce 50% growth inhibition in stationary culture growth experiments. However, at high concentrations cobalt does have an inhibitory effect on protein synthesis at the level of amino acid incorporation into protein, whereas nickel fails to reveal such an effect (Table i and fig III).

The striking increase in the levels of proteinase, acid and alkaline phosphatases with increase in the cobalt concentration of the growth medium is not observed when the organism is grown with corresponding

levels of nickel. Kuo and Blumenthol (8,9) have found the phosphatases of N. crassa to be non-specific, acting on atleast 20 phosphorylated compounds. However, it is held that factors other than the in vitro specificity of an enzyme may be operative in vivo and that the mold phosphatases may play a key role in the regulation of the levels of key phosphomonoesters participating in a wide variety of biochemical reaction cycles. Thus, the catabolic rate of these vital intermediates as well as the cell proteins may be considerably enhanced at concentrations of cobalt inducing 50% growth inhibition.

Both cobalt and nickel interfere with the metabolism of TCA cycle intermediates, but at different sites. These results are presented in Table III. The failure of keto acids to counteract growth inhibition due to cobalt is consistent with the observation of Dingle et al (12) in rat liver mitochondria that cobalt inhibits keto acid oxidation probably by forming a complex with the dithiol form of lipoid acid, a coenzyme for keto acid dehydrogenation. The enhanced uptake of cobalt in presence of these intermediates in N. crassa has also been observed in rat liver mitochondria. However, A. niger a citric acid accumulating strain, has been found to respond differently to the keto acids in cobalt toxicity, where the former can counteract the growth inhibition due to cobalt (13). In N. crassa the beneficial effects of citrate and malate can be attributed at least in part to their inhibition of the toxic metal uptake. The counteracting effects of pyruvate and α-ketoglutarate in nickel toxicity not accompanied by an inhibition of the toxic metal incorporation indicate a probable inhibitory effect of nickel at the level of formation of these ketoacids. Healy, Cheng and McElroy (5) have found that both cobalt and

nickel depress the levels of succinic dehydrogenase in N. crassa which can account for the failure of added succinate to counteract growth inhibition due to these heavy metals in this organism.

All these observations coupled with the demonstration that only cobalt toxicity corresponds to a straight iron deficiency in N.crassa calls for a general mechanism for the counteracting effects of iron and magnesium in not only in cobalt but also in nickel toxicity. Fig VII indicates that both iron and magnesium suppress the toxic metal uptake upto 24 hr of growth, but subsequently the inhibitory effect of iron is released and magnesium maintains its suppressing influence. These observations lend support to the suggestion (2) that iron transports the toxic metal to non-toxic sites. The toxic metal that accumulates after 24 hr of growth in presence of counteracting levels of iron may be at innocuous sites without impairing the formation and function of metabolites. Thus, iron may not only satisfy the deficiency status created, as for example in the case of cobalt, but may also have a more generalised mode of counteraction as discussed above, which can account for its beneficial effect not only in cobalt toxicity but also in those of nickel and zinc.

1. Toxic levels of nickel delay the initiation of mycelial growth. Nickel toxic mycelia contain significantly higher concentrations of protein as compared to those of cobalt-toxic or normal ones.

2. Neither cobalt nor nickel shows a specific inhibition of protein synthesis in preformed log phase normal mycelia, at concentrations which can induce 50% growth inhibition in growth experiments. At high concentrations an

inhibitory effect of cobalt on protein synthesis is evident at the level of amino acid incorporation into protein.

3. The mycelia grown with increasing levels of cobalt possess enhanced levels of proteinase, acid and alkaline phosphatases. Corresponding levels of nickel do not influence these enzyme activities.

4. Citrate and malate counteract growth inhibition due to cobalt and nickel. Pyruvate and α-ketoglutarate are beneficial in the case of nickel but not in the case of cobalt. Succinate and fumarate are not beneficial in either case.

5. Citrate and malate inhibit toxic metal uptake by the organism. Pyruvate and α-ketoglutarate strikingly enhance cobalt incorporation but have no influence on nickel uptake. Succinate and fumarate enhance cobalt uptake to a smaller extent.

6. Iron and magnesium suppress toxic metal uptake in stationary culture growth experiments till 24 hr of growth. Subsequently the inhibitory effect of iron is released but that due to magnesium is maintained throughout the growth period.

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General

1. The present investigation has been primarily directed to gain an of some aspects of iron metabolism in *N. crassa* by studying the cobalt-iron antagonism in this organism. A comparative study on the cobalt and nickel toxicities has also been carried out in this mold.

2. A new iron-binding compound (X) has been isolated from fluid when *N. crassa* is grown under cobalt-toxic conditions. An iron-binding compound is also secreted when the organism is grown under conditions of straight iron deficiency. Preliminary studies indicate that the two iron-binding compounds may be identical.

3. Degradative studies have been carried out on the iron-binding compound isolated as the iron-complex (XFe). These studies indicate that XFe contains δ-N-hydroxy ornithine and β-methyl glutaconic acid or a closely related compound. The quantitative estimation of the degradation products indicates that XFe can be distinguished from the other members of the siderochrome class of compounds though it is closely related to them.

4. The production of the iron-binding compound is determined by the iron status of the growth medium. Decresing levels of iron result in increasing production of the iron-binding compound and a corresponding fall in catalase activity. In iron deficiency, the production of the iron-binding compound precedes the fall in catalase activity, when these parameters are assessed as a function of the growth period. XFe acts as a good iron source for the organism to maintain normal growth, catalase activity and a few non-heme iron enzyme activities. XFe^{59} is incorporated at a faster rate as compared to $Fe^{59}Cl_3$ or Fe^{59}citrate, into the iron-deficient *N. crassa* mycelium.

5. Increasing levels of cobalt in the medium result in increasing production of the iron-binding compound and a corresponding fall in catalase activity. Cobalt does not inhibit iron incorporation into the mycelium from the medium. It accelerates the iron uptake of the organism and depletes the medium of iron at an early stage of growth. The manifestation of an intracellular cobalt-iron antagonism is evident by the decreased bound iron, heme iron and catalase levels when there is a build up of cobalt concentration at the competition sites. Here also, the primary manifestation of iron deficiency is the secretion of the iron-binding compound. The intracellular cobalt-iron antagonism is evident at the level of heme synthesis. Cobalt inhibits the synthesis of the porphyrin moiety and cell-free extracts inhibits iron incorporation into protoporphyrin when present in definite excess of iron. XFe^{59} acts as a better iron source for heme synthesis in cell-free extracts as compared to $Fe^{59}SO_4$

6. It is held that a chelate closely similar to XFe is formed in the cell when the organism is grown under normal conditions with optimal levels of iron. The iron-binding compound is secreted into the medium when iron is unavailable for the formation of the natural chelate. The rapid incorporation of XFe^{59} into the mycelium and its superiority over inorganic iron as an iron source for heme synthesis in cell-free extracts, the detection of the iron-binding compound in the culture fluid in iron deficiency (straight or induced by cobalt) before the other iron-dependent systems are affected and the other findings have all been taken to conclude that the formation of the natural chelate represents a very early phase in iron transport in this organism and has an important role to play as an iron

donor for key iron dependent systems.

7. A comparative study on cobalt and nickel toxicities in N. crassa has revealed that the primary effect of nickel is a delay in the of mycelial growth. Nickel toxic mycelia contain significantly higher concentrations of protein as compared to the cobalt-toxic or normal mycelia. Increasing concentrations of cobalt in the growth medium result in enhanced levels of degradative enzymes like a proteinase and acid and alkaline phosphatases in the mycelium. Increasing concentrations of nickel do not have such an effect. Keto acids like pyruvate and α-ketoglutarate can counteract nickel toxicity but not cobalt toxicity. It is held that nickel may manifest its toxic effect at the level of keto acid formation and cobalt may act at the site of keto acid oxidation. Iron counteracts both cobalt and nickel toxicities and it is indicated that iron may bring out a transportation of the heavy metal from the toxic to the non-toxic sites in addition to satisfying the deficiency status created as for in cobalt toxicity.

SUPPLEMENTARY CHAPTERS

Reprinted from ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, Volume 99, No. 2, November, 1962

The Effect of Some Organic Acids on Metal Toxicities in *Aspergillus niger*[1]

It has been recently demonstrated that in Co, Ni, and Zn toxicities in *Aspergillus niger*, the growth inhibition was accompanied by impaired carbohydrate metabolism as reflected by decreased capacity to utilize glucose and produce acid (1). Hence, it appeared likely that under conditions of metal toxicities, the metabolism of several organic acid intermediates of carbohydrate breakdown was affected. This communication deals with experiments designed to investigate this possibility.

The strain of *A. niger*, the basal medium, and the culture conditions employed was identical with those of the earlier study (1). The mold was grown for 5 days at 30 ± 1°C. on 10 ml. basal medium (pH 2.5–3.0) in 100 ml. pyrex conical flasks. Toxicities were induced by the inclusion of either 400 μg. Ni, 1000 μg. Zn, or 1200 μg. Co per 10 ml. of medium. Pyruvate was added aseptically to the autoclaved medium after separate sterilization by "Seitz" filtration. The other organic acids were included as aqueous solutions (pH 2.5–3.0) along with metals. At the end of the experiment, the mycelia were washed thoroughly, dried, and weighed. Total acid elaborated into the culture medium was estimated as described previously (1). In metal uptake studies, 14.89×10^3 counts/min. of Co^{60} (carrier-free) or 9.2×10^3 counts/min. of Zn^{65} (100 mc./g.) were included suitably with the respective metals. Zn and Co uptake were determined on the basis of mycelial radioactivity (2) and Ni by the colorimetric estimation by the dimethylglyoxime procedure (3). The results obtained are shown in Table I.

The levels of the organic acids mentioned are those giving maximum reversals in the parameters tested, with the exception of fumarate, the higher levels of which could not be included due to its comparatively low solubility. It can be seen that generally, these organic acids annual growth inhibition very appreciably in metal toxicities and that malate and pyruvate are the most effective in this respect. Co toxicity is exceptional in that citrate is beneficial only to a small extent and succinate brings about an added inhibition. Further, the amounts of total acid produced are also enhanced significantly. That these responses are due to

TABLE I

INFLUENCE OF SOME ORGANIC ACIDS ON METABOLISM AND METAL UPTAKE IN METAL TOXICITIES IN *A. niger*

Supplements to 10 ml. basal medium	Mycelial wt. (mg. dry)	Acid[a] formed	Metal uptake (μg. metal/100 mg. dry mycelium)
None (control)	182.0	20.2	—
Cobalt (1200 μg.)	70	2.0	125.1
Cobalt (1200 μg.) + citrate (200 mg.)	98	13.1	129.5
Cobalt (1200 μg.) + succinate (200 mg.)	39	12.5	126.3
Cobalt (1200 μg.) + fumarate (70 mg.)	108	1.8	129.2
Cobalt (1200 μg.) + malate (200 mg.)	155	11.4	126.0
Cobalt (1200 μg.) + pyruvate (200 mg.)	185	30.1	12.9
Zinc (1000 μg.)	72	2.2	105.2
Zinc (1000 μg.) + citrate (200 mg.)	133	15.0	191.4
Zinc (1000 μg.) + succinate (200 mg.)	139	10.5	167.6
Zinc (1000 μg.) + fumarate (70 mg.)	147	7.4	151.9
Zinc (1000 μg.) + malate (200 mg.)	204	10.7	60.2
Zinc (1000 μg.) + pyruvate (200 mg.)	183	28.0	17.4
Nickel (400 μg.)	71	2.2	43.0
Nickel (400 μg.) + citrate (200 mg.)	121	13.8	32.5
Nickel (400 μg.) + succinate (200 mg.)	136	11.1	36.0
Nickel (400 μg.) + fumarate (70 mg.)	174	7.6	42.5
Nickel (400 μg.) + malate (200 mg.)	186	12.0	41.4
Nickel (400 μg.) + pyruvate (200 mg.)	230	26.0	3.9

[a] Total acid: Equivalents (ml.) of 0.05 *N* NaOH per 2.0 ml aliquots culture medium.

[1] This work was conducted with the financial assistance from the Council of Scientific and Industrial Research, New Delhi, India.

interaction at the metabolic level is indicated by the magnitude of the changes observed and the fact that under these conditions, generally, there was no significant depression in the mycelial accumulation of the toxic metals. However, there is a very considerable suppression of toxic metal uptake with pyruvate in all toxicities studied and with malate in case of zinc toxicity. This is rather unusual, but the possibility of extracellular complex formation is precluded by the high acidity of the basal medium, which increases further as a consequence of acid accumulation. Moreover, when each of these acids was included singly in absence of toxic metals, at the levels tested, they did not cause any metal deficiency by complex formation.

In *A. niger*, under conditions where the conversion of sugar is partial, as in the present study, the formation of citrate involves a recycling of C_4-dicarboxylic acids (4) and supplementary mechanisms apart from those involved in a 100% conversion (5, 6) must be operative. Hence, the tricarboxylic acid cycle would not be completely blocked and an adequate supply of intermediates would be necessary for normal metabolism. On this basis, the results presented herein suggest that derangements in metal toxicities in *A. niger* involve principally the metabolism of the intermediates of carbohydrate degradation. The primary lesions are probably at the level of formation of pyruvate and malate. It is noteworthy, in this context that in *Neurospora crassa* (7), metal toxicities have been shown to depress succinic and isocitric dehydrogenase levels. The ineffectiveness of succinate in Co toxicity may be attributed to specific Co-Fe antagonism reported in *A. niger*, which differentiates it from Zn and Ni toxicities (1, 2) and may be at the level of succinic dehydrogenase. However, some features such as the general suppression of metal uptake induced by added pyruvate and the similar effect of malate in Zn toxicity are not easily explained and await further study.

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A Sensitive Microbiological Assay Procedure for determining Magnesium in Biological Materials

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A simple microbiological assay procedure for determining magnesium in the range 1 to 8 μg has been developed, in which *Neurospora crassa* Em 5297a is used as the test organism. For the range 1 to 4 μg of magnesium, an indirect assay procedure based on the determination of the mycelial content of the "phenol reagent - positive material" has also been evolved, the dry weight of mycelia being directly used as an index of response for higher levels of magnesium. The method has been found to be applicable to various biological tissues, the recoveries being about 95 to 105 per cent.

ATTEMPTS to develop microbiological methods for determining metals have so far been mainly unsuccessful. Manganese has been assayed with *Lactobacillus arabinosus* 17–5 in biological materials,[1] and *Aspergillus niger* has been used for determining several metals, including magnesium.[2] However, the *A. niger* procedure for determining magnesium requires a sample containing at least 50 μg of metal at a concentration of 1 μg per ml of culture medium. Macleod and Snell[3] studied the magnesium requirements of several *Lactobacilli* with a view to using them for the assay of magnesium, but found that, even with pre-treatment of a magnesium deficient medium with *Lactobacilli*, it was not possible to reduce growth in an unsupplemented medium. During earlier work with the mould *Neurospora crassa*[4] it was found that the wild strain of this mould, Em 5297a, had a low requirement for magnesium, and growth was totally absent if pure salts were used to constitute the medium and magnesium was omitted. Advantage was taken of this observation, and a sensitive microbiological assay procedure is described here, in which this mould is used as the test organism.

METHOD

APPARATUS—

The mould was grown in Pyrex-glass test-tubes (150 mm × 15 mm) on a reciprocal shaker (stroke 10·8 cm; 84 cycles per minute) in a constant-temperature chamber kept at 30° ± 1° C; the test-tubes were kept inside the chamber in a test-tube rack mounted so that the tubes were tilted, lips upwards, so as to spread the medium over a length of about 7 to 8 cm. After inoculation, the organism was grown with continuous shaking during the entire growth period.

Pyrex glassware was used throughout; it was cleaned with chromic - sulphuric acid, and then rinsed with distilled water, and, finally, with water distilled in an all-glass apparatus.

REAGENTS—

Acids—Analytical-reagent grade acids were used; the concentrated nitric acid was further purified by distillation before use.

Folin and Ciocalteu's phenol reagent—Prepared as recommended by Lowry, Rosebrough, Farr and Randall.[5]

ASSAY MEDIUM AND GROWTH TECHNIQUE—

The culture medium used in all assays was based on that used in earlier work[4] with the difference that magnesium sulphate was replaced by sodium sulphate to provide an equivalent amount of sulphur. The percentage composition was: glucose, 2; potassium dihydrogen orthophosphate, 0·3; ammonium nitrate, 0·2; ammonium tartrate, 0·1; sodium sulphate, 0·025; sodium chloride, 0·01; calcium chloride, 0·01.

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The trace elements supplied (in μg per 100 ml) were: zinc, 20; manganese, 20; copper, 8; iron, 2; molybdenum, 2. Biotin (0·5 μg per 100 ml) was added, and one drop of Tween 80 per 100 ml of medium was also added to inhibit sporulation.[6]

For assay purposes, the organism was grown on the above medium (adjusted to pH 4·8 to 5·0) for 72 hours at 30° ± 1° C with shaking as described above. A 2·0-ml portion of double-strength medium and 2·0 ml of sample in aqueous solution adjusted to the pH of the medium were taken in each assay tube.

STOCK CULTURE AND INOCULUM—

Neurospora crassa Em 5297a was used as the assay organism. It was maintained by transfer biweekly on agar slants of the above medium supplemented with 0·2 per cent. each of yeast and malt extracts and containing 2 per cent. of agar. For inoculation, spore from a 7-day-old culture was used, one drop of a suspension of spore having a transmission of approximately 90 per cent. in sterile water distilled in an all-glass apparatus being used in each tube.

ASSESSMENT OF GROWTH REPONSE—

At the end of the growth period, the mycelia were carefully removed, washed, and dried at 60° C overnight; the assay was then completed by determining the mycelial weight.

In assaying magnesium in the range 1 to 4 μg, the mycelia were carefully removed, rinsed on a filter-paper with water distilled in an all-glass apparatus, and transferred to Pyrex-glass test-tubes (100 mm × 10 mm). A 0·5-ml portion of 5 N sodium hydroxide was added to the contents of each tube, and the tubes were heated on a boiling-water bath for 30 minutes; they were then cooled, and the contents were adjusted to 5 ml with distilled water. Portions (0·5 ml) were removed, after centrifugation if necessary, for determining the "phenol reagent - positive material" according to the method of Lowry *et al.*,[5] a Klett-Summerson photo-electric colorimeter and a No. 66 red filter being used.

PREPARATION OF SAMPLE—

The biological sample was wet-ashed in a 50-ml Pyrex-glass conical flask with 10 ml of concentrated nitric acid and 1·0 ml of 66 per cent. perchloric acid (Merck G.R.) per gram of the sample on a sand-bath. After complete evaporation, the digest was treated with 5 ml of concentrated hydrochloric acid, and once again evaporated to dryness. To the residue dissolved in 5·0 ml of distilled water was added 0·1 ml of a 50 per cent. w/v solution of ammonium chloride, and then 0·05 ml of ammonia solution, sp.gr. 0·88, was added to make the solution alkaline. A 0·1-ml portion of a 0·5 per cent. solution of ferrous sulphate was added and hydrogen sulphide was bubbled through the solution for 15 to 20 minutes. After the solution had been set aside for 2 hours, the precipitated ferrous sulphide, together with sulphides of other heavy metals, if any, was removed by filtration through a fluted Whatman No. 42 filter-paper, and washed with 15 to 20 ml of distilled water. The filtrate and the washings were combined, and evaporated to dryness on a sand-bath, 1·0 ml of concentrated nitric acid being added during the later stages of the evaporation. The residue was dissolved in a known volume of distilled water, and adjusted to pH 4·8 to 5·0. Portions of this solution were used for the assay.

RESULTS

RANGE OF ASSAY—

Under the conditions of growth obtaining in the proposed procedure, when analytical-grade reagents are used and normal precautions are taken to eliminate magnesium as a contaminant, growth is negligible on the basal medium constituted without magnesium. The microbiological response curve obtained is shown in Fig. 1.

It will be seen that the useful range of assay is from 1 to 8 μg of magnesium. The mycelial weights have been found to be highly reproducible, variation between duplicates never exceeding 0·2 mg over the entire range.

For samples containing 1 to 4 μg of magnesium, the indirect method of estimating growth based on "phenol reagent - positive material" has been developed to ensure higher accuracy without involving elaborate manipulation. If the mycelia are pressed gently to dryness before suspension in sodium hydroxide, the alkali digest can be directly adjusted to volume by adding 4·5 ml of water, and portions can be removed for assay. Under the

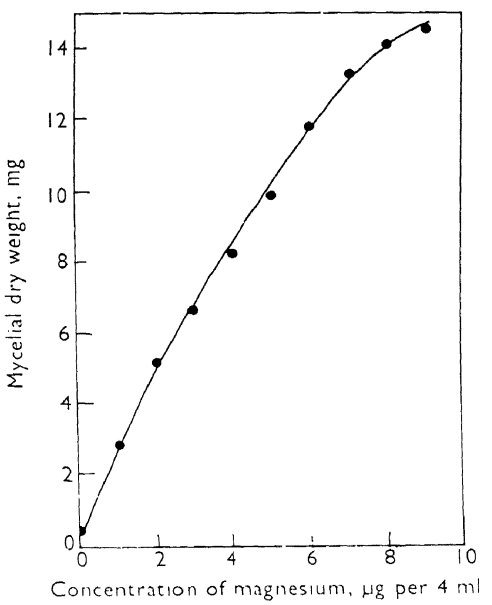
conditions proposed, we have found the extraction of "phenol reagent - positive material" is complete in one step. In the range 1 to 4 μg of magnesium, the "phenol reagent - positive material" gives a linear standard graph. Preliminary experiments showed that over 70 per cent. of the "phenol reagent - positive material" is protein. For samples containing less than 1·0 μg of magnesium, the microbiological assay procedure is not recommended, even with the indirect method based on "phenol reagent - positive material" of the mycelium.

TABLE I

APPLICABILITY OF THE *N. crassa* ASSAY PROCEDURE FOR DETERMINING MAGNESIUM IN BIOLOGICAL TISSUES

Sample	Portion taken for assay, ml	Magnesium found, μg	Total magnesium content, μg per g fresh weight	Magnesium recovered,* μg	Magnesium recovered, %
Tecoma stans leaves	0·2	2·50	625·0	3·50	100
	0·4	4·95	618·7	5·90	95
	0·5	6·20	620·0	7·10	90
L. sativus seeds ..	0·2	3·00	1500	3·95	95
	0·3	4·35	1450	5·30	95
	0·4	5·90	1475	6·90	100
Rat kidney ..	0·2	2·25	162·5	3·20	95
	0·4	4·60	166·1	5·55	95
	0·6	6·70	158·9	7·80	110
Rat blood	1·0	1·20	30·0†	2·20	100
	1·5	1·80	30·0†	2·75	95
	1·9	2·45	32·2†	3·40	95
Rat spleen.. ..	0·2	1·65	221·7	2·70	105
	0·4	3·40	228·5	4·40	100
	0·6	5·00	224·0	6·00	100
Rat liver	0·2	2·10	145·8	3·15	105
	0·4	4·30	149·3	5·25	95
	0·6	7·00	161·7	7·95	95

* Amount recovered after 1 μg of magnesium had been added.
† Expressed as μg per ml.



1. Microbiological assay curve for magnesium with *N. crassa*

Applicability of the method to biological tissues

The applicability of the proposed method has been studied for several biological tissues. Recoveries have been tested by adding magnesium at the 1·0-μg level to three different portions of each biological tissue tested. The absolute values and the mean recoveries obtained are shown in Table I, and represent values derived from at least four independent analyses for each type of sample. It can be seen that the method is suitable for the type of samples investigated throughout the concentration range.

Interference from other metals

The effect of other metals on the assay of magnesium by the proposed procedure has been studied. The metals mentioned below do not interfere in the assay up to the levels indicated (μg per tube): sodium, 3000; potassium, 51,000; calcium, 2100; iron, 1000; zinc, 10; cobalt, 10; nickel, 5.

Discussion of the method

The only useful microbiological method for determining magnesium so far has been that of Nicholas[2] in which *A. niger* is used. However, it involves the use of carefully purified media and a sample having a magnesium content of at least 50 μg. The considerably lower requirement for magnesium of *N. crassa* and the fact that elaborate purification of the assay medium is unnecessary are distinct advantages in the proposed method. Further, by use of a simple procedure for the indirect determination of growth reponse, it is possible to work conveniently with good precision at levels as low as 1 μg. The proposed procedure is also specific for magnesium, since calcium, sodium and potassium do not interfere, except at extremely high levels compared with the amount of magnesium present. Heavy metals, such as cobalt, nickel and zinc, which interfere with magnesium metabolism in the mould[4] and are often present in biological material in various amounts, were found to be completely eliminated by the hydrogen sulphide treatment introduced during the preparation of the sample. Since uniformly good recoveries were obtained for all the sample sizes with the various types of biological materials, the proposed procedure can be adopted with advantage, particularly since the conventional complexometric titration of magnesium[7] involves a correction for the presence of calcium and is of limited usefulness below 10 μg of magnesium.

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The Isolation of a Toxic Principle from *Lathyrus sativus* Seeds

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The insect larva *Corcyra cephalonica* St. has been found to respond to the toxic principles present in *L. sativus* seeds. A new ninhydrin-positive compound has been isolated from the seeds of *L. sativus* and some of the properties of the compound are reported. This compound is highly toxic to several microorganisms in minute amounts.

THE consumption of *L. sativus* seeds as the principal dietary constituent causes a neuropathological disease called 'lathyrism' among the rural population in certain parts of Central India[1]. Difficulty has been experienced in producing pathological symptoms characteristic of lathyrism in laboratory animals[2-4]. Uncertainty exists regarding the exact nature of the causative factors for the disease, though high contents of Se[5] and Mn[6] as well as low contents of methionine[7] and tryptophan[8] in the seeds have been suggested as possible etiological factors. The absence of alkaloids[9] and poisonous aminonitriles[10,11] in the seeds has also been reported. It is, therefore, of interest to examine other chemical constituents of the seeds for their biological activity. As a first step, the effect of *L. sativus* seeds on the insect larva *Corcyra cephalonica* St., an organism with nutritional requirements resembling those of higher organisms[12], has been investigated. In the present communication, the response of *Corcyra* to feeding of *L. sativus* seeds, and the isolation of a new ninhydrin-positive compound toxic to several microorganisms are reported.

Incorporation of *L. sativus* seed meal at 30 per cent level in the basal wheat flour diet[13] led to a marked growth inhibition to the extent of 60 per cent. A gross fractionation of the seed meal with different organic solvents was carried out to isolate the inhibitory factors. Ether and chloroform extracts failed to affect the growth of the organism while the seed residues still retained inhibitory properties. However, aqueous ethanol (75 per cent) extract proved deleterious to larval development, indicating the extractability of the toxic principles by aqueous ethanol. The seed meal residue allowed normal growth of the insect at comparable dietary levels.

In view of the reported presence of a pharmacologically active amine in *L. sativus* seeds[14], the alcohol extract was examined for the possible presence of unusual nitrogenous constituents such as rare amino acids. Paper chromatography on Whatman No. 1 filter paper using *n*-butanol-pyridine-water-acetic acid (4: 1: 1: 2; vol./vol.) revealed two prominent ninhydrin-positive components (R_f values 0·09 and 0·24) not corresponding to the usual amino acids found in proteins. The bulk isolation of these two constituents was, therefore, attempted and the isolation of one of them is described below. The isolation and characterization of the other compound will be reported elsewhere.

The seed meal (400 g.) was refluxed with 600 ml. of aqueous ethanol (75 per cent) for 90 min. and the extraction repeated thrice with fresh portions of the solvent. The pooled extract was filtered, concentrated *in vacuo* (40-45°C.) to one-sixth the volume and shaken with an equal volume of chloroform to remove lipids and pigments. The aqueous layer (*p*H 5-6) was passed through a column of Dowex-50 × 8 (200-400 mesh; resin volume 117 ml.) in the H^+ form. The break-through fluid containing starch and sugars was rejected and the column washed with 1 litre of distilled water. The effluent contained only the ninhydrin-positive component (R_f value 0·09) and the washing continued to secure its complete elution. The eluate was concentrated by freeze-drying to one-fourth the volume in a 'Virtis' freeze mobile and the compound precipitated by addition of acetone and purified by repeated acetone precipitation from aqueous solutions; yield 0·8 g.

The acidic character of the isolated product was suspected by its behaviour on the Dowex-50 column and an aqueous solution (2 mg./ml.) had a *p*H of 2·5. The compound reduced the Folin-Ciacalteu reagent[15] and could be quantitatively estimated by the method of Lowry *et al.*[16], the colour intensity per mg. being equivalent to that due to 0·04 μmole of tyrosine. When treated with ninhydrin according to Rosen[17] the colour yield obtained per mg. was equivalent to that due to 5·35 μmoles of leucine.

The homogeneity of the isolated compound was established by paper chromatography in 4 different solvent systems and the R_f values obtained are given in Table 1. Further, when adsorbed on a column of Dowex-1 × 8 (200-400 mesh) in CH_2ClCOO^- form and

TABLE 1 — R_f VALUES OF ACIDIC COMPOUND

Solvent system	Chromatographic technique	R_f value
n-Butanol-acetic acid-water (4:1:1)	Circular	0·15
Pyridine-water (80:20)	do	0·45
Phenol-water (80:20)	Ascending	0·14
Aqueous ethanol (75%)	Circular	0·50

eluted with 0·1*M* $CH_2ClCOONa$, the elution pattern also showed a single symmetrical peak in conformity with the paper chromatographic data.

On treatment with 6*N* HCl or a saturated solution of $Ba(OH)_2$ at 105°C. for 24 hr, the compound yielded another ninhydrin-positive substance (R_f value 0·28) on circular paper chromatograms with *n*-butanol-acetic acid-water (4:1:1) as the solvent. The latter, unlike the parent substance, could be adsorbed on Dowex-50 and eluted with 2*N* HCl. When treated with periodate reagent[18], ammonia could be detected on spraying with Nessler's reagent. It also fluoresced under ultraviolet light on treatment with acetylacetone[19] unlike the original compound. These reactions indicate the presence of an amino and a hydroxyl group in juxtapositions in this product. On continuing the acid or alkali treatment for 72 hr two additional ninhydrin-positive bands (R_f values 0·38 and 0·46) were obtained on paper chromatograms with the above solvent system.

The isolated compound inhibited the growth of *Neurospora crassa* (wild, Em 5297a) by 50 per cent at a concentration of 4 μg./ml. and completely at 8 μg./ml. of the basal medium when the mold was grown on the minimal medium[20] for 72 hr at 30°±1°C. with shaking. Preliminary data indicated that casein hydrolysate at 250 μg./ml. level counteracted the toxicity to a marked extent. The growth of *Staphylococcus aureus* N-15, *Escherichia coli* N-52 and *Candida albicans* Z-247 was also inhibited (about 50 per cent) by the compound at 7 μg./ml. and completely at 15 μg./ml. of the culture medium[21] when the organisms were grown for 24 hr.

The data obtained rule out the possibility of this compound being identical with those recently isolated and characterized as lathyrin and homoarginine from the seeds of several *Lathyrus* species[11,22] (also Rao, S. L. N., Ramachandran, L. K. & Adiga, P. R., unpublished data). Further work is in progress to characterize this compound and to study its effects on other organisms.

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FOLIN-CIOCALTEU REAGENT FOR THE ESTIMATION OF SIDEROCHROMES

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The isolation of a large number of iron-binding or iron-containing like ferrichrome, ferrichrome-A, ferrioxamines, terregens factor, and others (1,2) from microorganisms has led to investigations relating to the transport mechanisms involved in microbial iron metabolism. Recently a new iron-binding compound was isolated from Neurospora crassa (3). The production of these iron-binding compounds under conditions of iron deficiency and the metabolic potency of a representative compound like ferrichrome in controlling the heme synthesis in an organism like Arthrobacter JG 9 (4 - 6) have all been taken as evidences for the key role of these compounds in the metabolism of iron in these microorganisms. Another significant fact is that several of the iron-binding compounds isolated under iron-deficient conditions show a basic similarity to ferrichrome in possessing a hydroxamate structure at the iron-binding site and the ferric polyhydroxamate class of compounds have been referred to as siderochromes (7).

Earlier Neilands (8) described a method for the estimation of the ferrichrome based on the autooxidizable character of the reduced colorless form to the colored natural complex having an absorption maximum at 425

In the present investigation it has been found that and related compounds react with Folin-Ciocalteu reagent, under conditions

described to estimate protein (9), to give a blue complex which can be measured colorimetrically and the ferrichrome concentration assessed.

EXPERIMENTAL

Preparation of reagent and Assay Method. Folin-Ciocalteu reagent was prepared and the assay conducted essentially according to Lowry, Rosebrough, Farr and Randall (9). A 1.0 ml aliquot of sample containing 5-50 µg in the macro range and 0.2 ml aliquot containing 1-10 µg ferrichrome in the micro range were employed. The final color is measured in a Klett-Summerson colorimeter with filter No. 66 in the macro method; in the micro method the measurement is made in a 1 cm. cell with a Bausch & Lomb spectronic colorimeter at 660 mµ or at 750 mµ.

Preparation of the sample. Ferrichrome is the iron-containing compound present in the cells of Ustilago sphaerogena. Ferrichrome A is the iron-binding compound secreted into the culture fluid of the same organism, under iron-deficient conditions. A similar situation is realized in N. crassa as well, where the normal mycelia contain an iron-containing component having a close resemblance to the iron-binding compound isolated from iron-deficient culture fluid of the same organism. The sample preparation for estimation is essentially according to Neilands (8) and has been tested with N. crassa.

Briefly, the iron-containing compound is isolated from N. crassa mycelia by phosphate buffer extraction (pH 7.0). The phosphate buffer extract and the buffer wash are pooled after centrifugation and saturated with ammonium sulfate. A 3 ml aliquot of the supernatant is shaken with 1 ml of benzyl alcohol and centrifuged. A 0.5 ml aliquot of the benzyl alcohol layer is then shaken with 1:10 water-ether mixture and the water layer drawn and collected. The ether-benzyl alcohol layer is then washed with 1 ml of

water and the aqueous layers pooled and concentrated. A known aliquot is then used for estimation by the Folin method.

In the case of the culture fluid containing the iron-binding compound, a 2 ml aliquot is treated with 1 ml of iron solution (1 mg/ml) and centrifuged. The reddish colored clear supernatant is saturated with ammonium sulfate and the rest of the procedure is the same as indicated earlier.

RESULTS

Figure 1, which represents the standard curve obtained for ferrichrome in the range 5-50 µg, is found to exhibit a linear relationship. The effect of copper on the color yield has been tested in this range. It is found that the omission of copper from the alkaline reagent results in a fall in the final color yield, though the linearity is maintained. A similar linearity is demonstrable in the case of the iron-binding compound from N.crassa as is evident from Table 1. The values obtained with a standard protein like bovine serum albumin have also been given and the siderochromes give greater color yields on weight basis. Ferrichrome A falls in line with the other two, though the final color yield obtained with each compound may be different.

Table 2 indicates the linearity obtained in the range 1-10 µg of ferrichrome when estimated by the micromethod. In all cases the final color yield obtained remains almost a constant from 30 to 60 min after addition of the Folin reagent.

Hydrazine and hydroxylamine do not give a detectable color in the range 5-50 µg but aceto- and benzohydroxamic acids give an appreciable reaction, as is evident from the absorption curves depicted in Fig. 2.

Fig.I

Standard curve for Ferrichrome estimation using Folin-Ciocalteu reagent

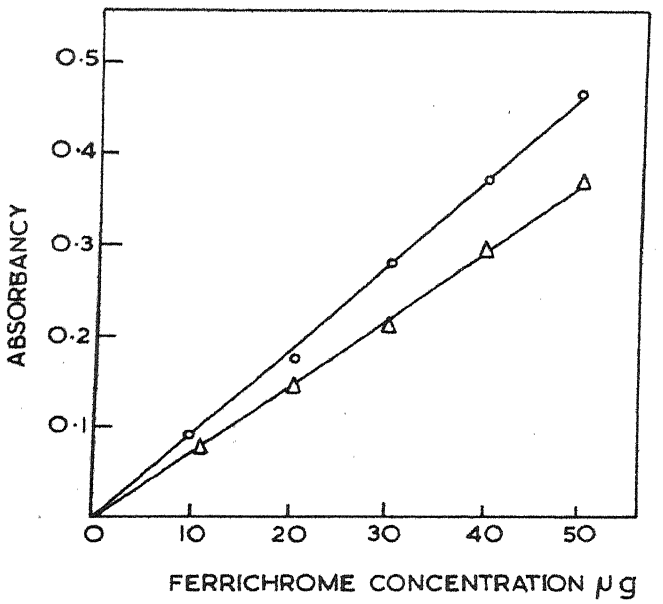

Fig.II

Absorption spectrum of the hydroxamate complexes with Folin-Ciocalteu reagent

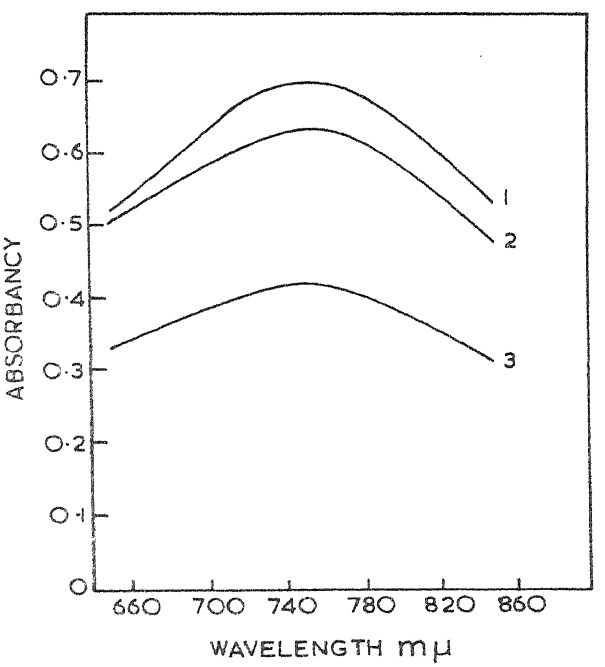

TABLE 1

Estimation of the N. crassa iron-binding compound by the Folin method

Experimental details are given in text

Concentration of compound µg/ml	Absorbancy	
	N. crassa compound	Bovine serum albumin
10	0.062	0.024
20	0.128	0.048
30	0.186	0.068
40	0.254	0.096

TABLE 2

Estimation of Ferrichrome in the Micro range by the Folin Method

Experimental details are given in text; absorbancy values reported are those obtained at 660 mμ

Concentration of ferrichrome μg/0.2 ml.	Absorbancy
1	0.046
2	0.086
4	0.168
6	0.271
8	0.357
10	0.456

TABLE 3

Recovery of added Ferrichrome and *N. crassa* Iron-binding Compound to culture fluid

was grown in 10 ml media in 50 ml pyrex conical flasks in stationary culture for 72 hr under normal and iron-deficient conditions. Normal mycelia were pooled and 10 gm of fresh mycelia extracted with 25 ml of phosphate buffer (pH 7.0); 3 ml was taken for iron-containing compound estimation. In iron deficient cultures, 2 ml of the culture fluid was taken for iron-binding compound estimation. Recovery values are reported after doing a paper chromatography of the final preparation obtained as indicated in the text.

Source	Absorbancy	
10 µg ferrichrome (A)	0.088	-
10 µg *N. crassa* compound (B)	0.062	-
3 ml mycelial extract	0.072	-
3 ml mycelial extract + (A)	0.156	95.4
3 ml mycelial extract + (B)	0.131	95.0
2 ml culture fluid	0.252	-
2 ml culture fluid + (A)	0.334	93.2
2 ml culture fluid + (B)	0.311	95.1

Iron-free ferrichrome (10) gives the same color yield as that of the intact ferrichrome. Similarly, the color yield obtained for aceto- and benzohydroxamic acids is not altered when they are used as ferric chelates.

The applicability of the method to biological material has been tested with N. crassa. Aliquots of phosphate buffer extracts of normal mycelia and culture fluid, when the organism is grown under iron-deficient conditions, have been processed as described in the experimental section. Recovery experiments have been performed by adding known amounts of ferrichrome and the N. crassa iron-binding compound to the phosphate buffer extracts as as to the culture fluid.

The final preparation from the mycelia has been found to contain traces of amino acids which, however, do not interfere in the present system. If it is found that the interference may be appreciable in certain other biological samples, the final preparation may be chromatographed on Whatman No.1 paper and developed with 50% methanol as solvent. In this system the siderochromes have generally high R_f values and can be easily detected as faint reddish spots to the naked eye or as dark spots in UV light, at 10 µg level. The spot can be cut and eluted with 50% methanol and an aliquot used for estimation. It is seen from Table 3 that the method can operate with an overall recovery of 95%.

DISCUSSION

It appears that the main basis of the procedure rests on the interaction of the hydroxamic acid with the Folin reagent to give a blue complex under alkaline conditions. Neither ferrichrome nor ferrichrome A, whose structures have been established (10,11), contain any of the amino acids such as tyrosine which form the basis for Folin color reaction of proteins.

The basic similarity between the siderochromes is the presence of a hydroxamate structure and all the three siderochromes tested as well as aceto- and benzohydroxamic acids answer the Folin reaction. The disproportionately high color yields of ferrichrome as compared to the aceto- and benzohydroxamic acids on a molar basis as is evident from Fig. 2 is striking. This may be a characteristic of the siderochrome molecules, with their polyhydroxamate character being a contributory factor. However, the method is reproducible with each type of hydroxamate compound. It is interesting to note that hydrazine and hydroxylamine fail to give any color in the range of the hydroxamic acids though at higher concentrations they also interact.

The advantages of the method are its simplicity and sensitivity. For the method based on oxidation-reduction reaction of ferrichrome (8) estimations are possible in the range 30-60 µg and it is more elaborate in manipulation. In the Folin method of estimation concentrations as low as 5 µg in the macro range and 1 µg in the micro range can be accurately determined.

Interfering materials in biological samples, generally proteinaceous in nature, are completely removed by ammonium sulphate saturation and benzyl alcohol extraction and, if necessary, by a subsequent paper chromatography. The entire procedure works out with an over-all recovery of 95%.

1. A simple method has been devised for the estimation of siderochromes based on their reaction with Folin-Ciocalteu reagent to give a blue complex under alkaline conditions.

2. The applicability of the method to biological systems has been tested with N. crassa and concentrations in the ranges 5-50 µg and 1-10 µg

can be accurately estiamted with an over-all recovery of 95%.

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